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13. ABSTRACT *(Maximum 200 Words)*

Purpose: Because of the potential synergistic interaction between an anti-angiogenic aminosterol, squalamine, and other angiogenic modifiers such as vascular endothelial growth factor (VEGF) and cytokines that may be released during intermittent androgen withdrawal therapy, we tested extensively the interaction between squalamine and VEGF for an enhanced cytotoxicity to human prostate cancer cells in vitro and xenografts tumor models in vivo. While in vitro synergistic interaction was demonstrated specifically in human prostate cancer cell lines containing a functional androgen receptor, we encountered difficulty in demonstrating such synergism in vivo for the reason that severe toxicity was noted when VEGF was delivered as an Ad-CMV-TK vector. For this reason, we explored the other possible synergistic interaction between squalamine and castration. **Results and Discussion:** Squalamine is highly synergistic to castration-induced endothelial destruction when applied at the time of castration. We noted VEGF receptor, flt-1 and integrin profile (e.g. $\alpha 6\beta 4$) can predict squalamine response. Prostate cancer cells lacking the expression of these markers may be less responsive to the synergistic interaction between squalamine and castration. We are currently assessing the possible interaction between squalamine and VEGF and squalamine and androgen status of the cell culture and in animals subjected to castration to evaluate if synergism may exist particularly against the growth of endothelial cells.

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Introduction:

The objective of this proposal is to seek for a combination therapy between a low molecular weight aminosterol squalamine which has anti-angiogenic activity against induced endothelial proliferation and migration and vascular endothelial growth factor, VEGF, on the growth of human prostate tumors both in vitro and in vivo. Although both agents when applied alone have little anti-tumor effect, they have remarkable synergistic action when applied together in tumor cells that express certain profiles of integrin isotypes and VEGF receptors. This approach is taken because of the known inherent genetic stability of endothelial cells which are required for tumor cells' continued growth and expansion and the potential clinical application of an effective combination therapy targeted at tumor and its endothelial supplies for the effective treatment of hormone refractory prostate cancers.

Body:

Task 1: Establishment of in vivo human prostate tumors:

Since the funding of this proposal, we have perfected the models of human prostate cancer progression using LNCaP cells as the starting cell lines (Thalmann, et al. 2000, Appendix 1).

Task 2: Construction, characterization and production of adenoviruses that contain VEGF driven by a CMV universal promoter:

The synergism of anti-tumor effect between squalamine and VEGF in vitro prompt us to test if these agents when applied together in vivo may have synergistic anti-tumor effect. Because of the inherent toxicity and expense of VEGF to inject intraperitoneally into mice bearing prostate tumors, we have constructed, characterized and tested an adenoviral (Ad) vector with the expression of VEGF under the control of a universal promoter, CMV. Results of these studies are presented below:

(a) *In vitro* studies: LNCaP and C4-2 cells were grown in culture. After becoming established, they were treated with a dose-response of squalamine (at 0.0, 10, 20, and 50μgms/day) and an Ad-CMV-VEGF construct (at MOI of 0.0, 0.5, 1.0 2.5, 5, and 10). An additional control group of squalamine+VEGF (at 5, 10, and 20μg/ml) was established.

In preliminary studies, as expected, neither squalamine nor VEGF alone had any growth inhibitory effect with the exception of a high dose of squalamine at 50μg/day. Conversely, also as expected, the combination of the two agents did result in a synergistic cell kill, lowering the effective killing dose of squalamine to 10-20μg/day.

By itself, Ad-CMV-VEGF when applied alone at 0.5, 1.0, and 2.5 MOI had little effect on prostate tumor cell growth in vitro. At 5 and 10 MOI, there was an increasing baseline toxicity and cell-kill. A synergistic effect of squalamine and Ad-CMV-VEGF was noted when cells were exposed to Ad-CMV-VEGF at 0.5, 1.0, or 2.5 MOI plus squalamine where we noted that squalamine exerted cytotoxicity when given as a

threshold dose (20μg/ml).

(b) *In vivo* studies: In pilot study, five athymic mice were implanted with LNCaP or C4-2 cells. They were treated with either saline of squalamine (20μg/day) and Ad-CMV-VEGF (MOI 2.5) injected directly into the tumor. The protocol was designed to inject twice with the adenovirus, at days 1 and 14. However, due to severe morbidity, the mice did not survive long enough for the second injection. There appears to be rapid and accelerated tumor volume increment in these mice with severe morbidity (weight loss, failure to thrive, etc.) within days of the first Ad-CMV-VEGF injection. Several mice decompensated so rapidly, that formal necropsy was impossible. Of the few mice in which tissue was available for analysis, gross inspection of the tumor demonstrated a bloody and highly vascular mass, with no capsule and little organized structure. Formal sectioning was not possible, given the amorphous nature of the tumor mass. Touch preps demonstrated little structural organization. These results suggest that VEGF synergism in vivo with squalamine requires further evaluation with regard to the toxicity of VEGF and the determination of the optimal dose of VEGF delivered as an Ad vector.

Task 3: Evaluation of the in vitro and in vivo synergism between squalamine and VEGF (or castration), and assessment of the biochemical and morphologic changes of the prostatic tissues in vivo.

1. Squalamine and VEGF exerted synergistic inhibitory action on the growth of prostate cancer cells.

(a) *In vitro* studies: Squalamine inhibits prostate cancer cell growth *in vitro*. The anti-proliferative effect of squalamine was evaluated in LNCaP and C4-2 human prostate cancer cells *in vitro* by [^{3}H]-thymidine incorporation [see Figure 1]. As a single agent, squalamine had no effect on growth of either cell line. The combination of both squalamine and VEGF, however, repressed LNCaP and C4-2 growth. Subsequent studies demonstrated that squalamine's cytotoxic activity was potentiated by VEGF only in LNCaP and C4-2 prostate tumor cells, which express flt-1. This effect was not seen in PC-3 and DU-145 prostate tumor cells, which lack flt-1. From these data we hypothesize that squalamine activity is dependent upon flt-1 expression, which is increased by exogenous VEGF. Androgen receptor positive but not negative prostate cancer cells are responsive to the combined growth inhibition between squalamine and VEGF.

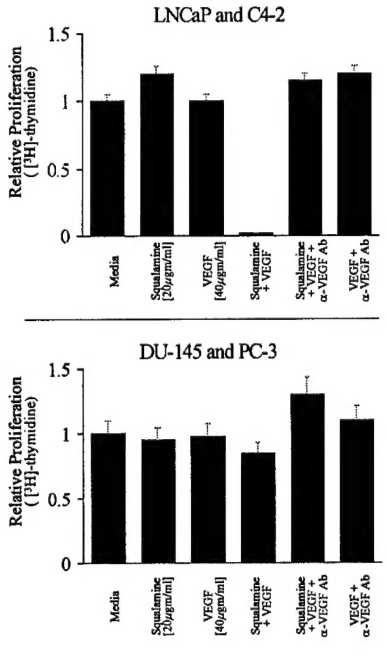

Figure 1: ***Top:*** At sub-cytotoxic doses of squalamine, LNCaP and C4-2 cells were killed *in vitro* when VEGF was added to the cultures. ***Bottom:*** This was not seen in DU-145 or PC-3 cells which lack flt-1 receptor expression.

(b) *In vivo* studies: Because of the in vitro results and the in vivo toxicity we observed of the VEGF, we decide to pursue the combined effect of squalamine and androgen ablation on tumor regression *in vivo*. The rationale of this approach is that androgen ablation

cause destruction of endothelium and the combined application of squalamine and androgen ablation may enhance an accelerated tumor death due to reduced in-growth by neovascular endothelium to tumor epithelium. To evaluate the effectiveness of squalamine *in vivo* in hosts undergo castration, androgen-responsive LNCaP tumors were first inoculated subcutaneously in intact male athymic mice. Mice that developed measurable tumors and had elevations in serum PSA were castrated, with a resultant fall in PSA to zero [see Figure 2]. The mice were divided into three groups.

1. Control (n=5): Within 3 weeks after castration, the PSA began to rebound and tumor size, which plateaued after castration, began to increase concomitant with the PSA recurrence. The tumor size and PSA continued to rise unabated until the animals succumbed to their cancers.

2. Concomitant treatment (n=14): Squalamine was applied coincident with castration. In these mice, the PSA nadir was maintained for the duration of squalamine application, with concomitant tumor involution. The mice were followed for an average of 12 weeks. In 10 mice (henceforth, "responders"), tumors were reduced to small nubbins of scar-tissue, (a response rate of 71%). Immunohistochemical staining of tumor remnants showed strong integrin $\alpha_6\beta_4$ and flt-1, and little VEGF and integrin $\alpha_V\beta_3$ and $\alpha_V\beta_5$ expression [see Figure 3]. Conversely, non-responding mouse tissue stained strongly for integrin $\alpha_V\beta_3$ and $\alpha_V\beta_5$, with little integrin $\alpha_6\beta_4$ and flt-1.

3. Delayed treatment (n=12): Squalamine was applied ***after*** the post-castration rebound in tumor growth and serum PSA, neither of which was affected by the addition of squalamine. These tumors stained strongly for integrin $\alpha_V\beta_3$ and $\alpha_V\beta_5$, with little integrin $\alpha_6\beta_4$ and flt-1 expression [data not shown].

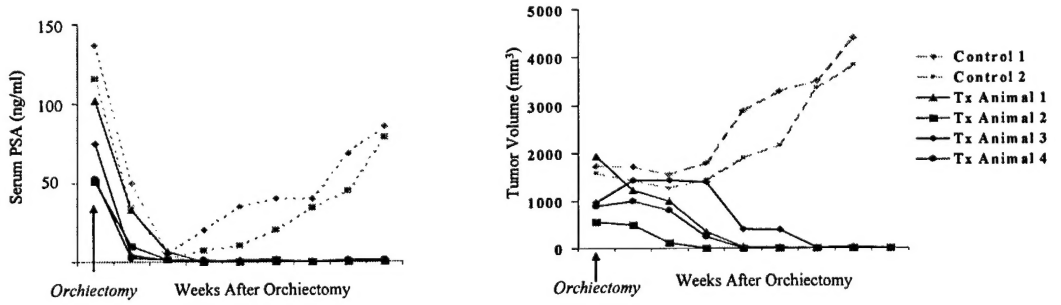

Figure 2: *Representative* graphs of *in vivo* data presented in text (above). PSA values *(left)* and tumor volumes *(right)* in 2 of the control (n=5) and 4 of the concomitant squalamine and castration mice (n=14) with established subcutaneous LNCaP tumors. After eight weeks the tumors measured approximately 1 x 1 cm and the mice underwent orchiectomy. In control mice *(dashed lines)*, after castration, PSA levels nadir and tumor volumes plateau. After several weeks, hormone-refractory elements become established and proliferate, and the mice ultimately succumb to prostate cancer. When squalamine is applied coincident with castration *(solid lines)*, the PSA nadir is prolonged and the tumors regress. Delayed treatment cohort mice are not shown -- their graphs replicate those of the control animals depicted above.

Immunohistochemistry: We theorize that androgen ablation induces apoptosis of both prostate cancer and stromal cells and while simultaneously causing an acute decrease in VEGF and increase in flt-1 within the prostatic stromal-epithelial milieu. These effects thereby potentiate squalamine's activity: inhibition of integrin $\alpha_V\beta_3$ and $\alpha_V\beta_5$. The

combination of these effects induces tumor regression.

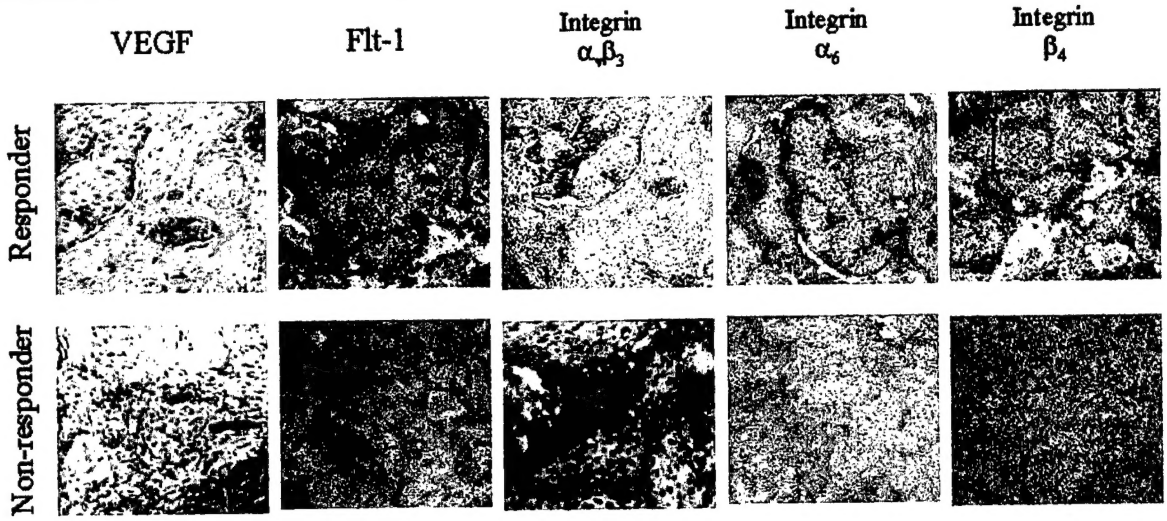

Figure 3: Expression of VEGF, Flt-1, and Integrins αvβ3, α6, and β4 in mice responding (*top*) and non-responding (*bottom*) to the combined therapy of castration and squalamine.

Task 4: Determine the in vitro effect of squalamine and /or VEGF on the growth of prostatic and endothelial cells.

We have completed the study of the combined growth inhibition by squalamine and VEGF in prostate cancer cell lines (see Figure 1 above). We are now testing the similar effect of these combinations on the growth of human endothelial cells.

Task 5: Recording of the morphologic changes of cells after squalamine and/or VEGF treatment.

Combined effects of squalamine and VEGF caused prostate cell apoptosis with typical nuclear chromatin condensation and DNA fragmentation. The combined effect of squalamine and androgen withdrawal on prostate tumor growth is influenced by the timing *when* squalamine was applied after androgen withdrawal. Histomorphologic and immnohistochemical features of the prostate tumors are presented in figure 3 above.

Task 6: Evaluation of the relationship between morphologic changes of prostate cancer and endothelial cells in vitro after squalamine and/or VEGF treatment with that of their biochemical expression of TSP-1 and cell surface integrin isotypes.

We have completed the analysis of morphologic and biochemical features of prostate tumors subsequent to squalamine treatment in intact and castrated hosts. This approach was taken because of the toxicity of VEGF observed when delivered directly into the tumors by Ad-CMV-VEGF vector (see above Task 2). We have also examined the expression of integrins by immunohistochemistry in squalamine responder and non-

responders (see Figure 3). In the future studies, we will evaluate the expression of TSP-1 and integrins in endothelial cells treated with squalamine and androgen withdrawal.

Task 7: Confirmation of the above biochemical responses of prostate cancer cells and endothelial cells to squalamine and VEGF in vivo.

As mentioned above, because of the significant toxicity we have experienced with in vivo application of VEGF, we re-directed our hypothesis to test the accelerated prostate cancer and endothelial cell death using the combinations of squalamine and androgen withdrawal. We are presently checking to see if endothelial cell toxicity can be augmented by the combined application of VEGF and squalamine.

Task 8: Evaluation of methodologies for evaluating signal cascade and apoptosis following VEGF and squalamine.

We have established the method to evaluate the immunoprecipitable focal adhesion kinase (pp125FAK) and it close family Pyk2 kinase in LNCaP and C4-2 cells. While the basal level of these kinases are similar among the cell lines, their status of phophorylation differs. In addition, we also have established the methods for apoptosis screen after cells are triggered to die under the influence of androgen withdrawal (Figure 4). These methods will be applied to evaluate the combined effects of squalamine and VEGF in vitro and the combined effects of squalamine and androgen withdrawal in vivo.

Task 9: Evaluation of changes in signal transduction components following exposure to squalamine and /or VEGF in vitro and confirmation of such changes in prostate tumor models in vivo.

This task will be carried out in this year since the basic methodologies applicable to this task have already been established.

Task 10: Characterization of changes of signal transduction components and their relationship to apoptosis, and comparison of their activity both in vivo and in vitro.

Basic methodologies used to address this task have been established. We will address this task question this year.

Key Research Accomplishments:

- VEGF delivered in the form of Ad-CMV-VEGF to prostate tumor-bearing mice was found to be extremely toxic. We saw increased blood perfusion and increased tumor volume upon Ad-CMV-VEGF treatment. Because of these in vivo findings, we have modified our procedure to test if destruction of blood vessel in animals maintained under castrated state can be enhanced upon rapid or delayed squalamine administration. Results of these study clearly demonstrated that squalamine can enhance castration-induced prostate tumor cell death if applied immediately after castration. Delayed application of squalamine after

castration resulted in rebound tumor growth suggesting that rapid alteration of sensitivity of blood vessels to squalamine during the early period of castration. Thus this model may shed light on cell signaling pathway in prostate cancer cells maintained either short-term or long-term under androgen withdrawal condition.

- We observed integrin profiles and VEGF receptor flt-1 are vastly different in tumors responded to combined effects of castration and squalamine and those did not. These differences may explain the type of integrin-extracellular matrix signaling system that may play a role in accounting for enhanced tumor response to combined squalamine and castration induced cytotoxicity in prostate cancer cells. Preliminary results suggest that $\alpha 6\beta 4$ and VEGF receptor flt-1 and their downstream signaling may assume important role.

Reportable Outcomes:

1. A joint publication with Dr. Mitch Sokoloff from University of Chicago is in preparation.
2. A published manuscript indicates the growth and metastatic potential of a human prostate tumor model (see Appendix 1 Thalmann, et al. Prostate, 2000).
3. A review dealing with prostate tumor-stroma interaction and the role of VEGF and hydrogen peroxide as modulators in the vicious cycle between tumor and stroma was conceived through the support of the present work (see Appendix 2 Sung and Chung, J. of Differentiation, in press).

Conclusions:

VEGF and squalamine synergism appears to be a phenomenon in vitro and its in vivo synergism is more difficult to demonstrated due to severe toxicity of delivery of VEGF to tumor tissues in tumor-bearing animals. The concept to enhance tumor and endothelial cell death using angiogenic modifiers however received support by the application of squalamine immediately after castration. Based on immunohistochemical data, it appears that tumor cells overexpress VEGF receptor, flt-1 and specific integrin isotype, such as $\alpha 6\beta 4$, are responders. This part of the work is currently pursued in Dr. Mitch Sokoloff's lab with addition of radiation and squalamine as a new combination. This work will be further explored and will be the subject of a future human clinical trial.

References:

None

Appendix:

1. Thalmann, et al. Prostate, 2000.
2. Sung and Chung, Journal of Differentiation (2002, In Press).

The Prostate 44:91–103 (2000)

LNCaP Progression Model of Human Prostate Cancer: Androgen-Independence and Osseous Metastasis

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BACKGROUND. Clinically, the lethal phenotypes of human prostate cancer are characterized by their progression to androgen-independence and their propensity to form osseous metastases. We reported previously on the establishment of androgen-independent (AI) human prostate cancer cell lines derived from androgen-dependent (AD) LNCaP cells, with androgen independence defined as the capability of prostate cancer cells to grow in castrated hosts. One of the sublines, C4-2, was found to be AI, highly tumorigenic, and metastatic, having a proclivity for metastasis to the bone.

METHODS. We established the AI and bone metastatic cell sublines B2, B3, B4, and B5 from the parental C4-2 subline, using a previously established coinoculating procedure. We determined the biologic behavior of the parental and derivative LNCaP sublines in vivo and in vitro, as well as their molecular and cytogenetic characteristics.

RESULTS. Unlike other human prostate cancer models, the LNCaP progression model shares remarkable similarities with human prostate cancer. We observed a comparable pattern of metastasis from the primary to the lymph node and to the axial skeleton, with a predominant phenotype of osteoblastic reaction; 25–37.5% of the animals developed paraplegia. Cytogenetic and biochemical characterizations of LNCaP sublines also indicate close similarities between human prostate cancer and the LNCaP progression model. Additional chromosomal changes were detected in B2–B5 sublines derived from C4-2 bone metastases. These LNCaP sublines were found to grow faster under anchorage-dependent but not -independent conditions. The in vitro invasion and in vivo metastatic potential of these LNCaP sublines surprisingly correlated with anchorage-dependent and not -independent growth. The derivative LNCaP sublines when cultured in vitro produced a substantially higher (20–30-fold) amount of basal steady-state concentrations of PSA than that of the parental LNCaP cells. PSA production was high initially, but was markedly reduced when the derivative cell lines were inoculated and allowed to grow long-term in vivo for the establishment of tumors and metastasis, suggesting that unknown host factors derived either from the prostate or the bone can effectively downregulate PSA expression by prostate tumor epithelium.

CONCLUSIONS. The LNCaP model of human prostate cancer progression will help improve

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our understanding of the mechanisms of androgen-independence and osseous metastasis, and tumor-host determinants of PSA expression. *Prostate 44:91–103, 2000.*
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KEY WORDS: **prostate cancer progression; androgen-independence; stromal-epithelial interaction; skeletal metastasis; PSA expression; cytogenetics; CGH; chromosomal losses and gains**

INTRODUCTION

The last decade has brought increased attention to and awareness of prostate cancer as a significant public health problem [1]. Prostate cancer is recorded as the leading cancer diagnosed and the second cause of cancer death in North American men [2]. Observations at autopsy indicate that early prostate cancer evolves in a multifocal pattern within the gland. Although the treatment of localized disease has significantly improved, once prostate cancer progresses to the periprostatic space by penetration and perforation of the prostate capsule and/or by invasion of the perineural spaces to the lymph nodes, few therapeutic options with limited durability are available. Clinical evidence suggests that after an initial responsiveness to androgen withdrawal, prostate cancer relapses to an androgen-independent state and metastasizes nonrandomly to the bone.

Little is known about the biology of prostate cancer metastasis, the underlying mechanisms of metastasis and androgen-independence, and the reciprocal interactions between prostate cancer epithelial cells and their surrounding stromal cells. This is due mainly to the fact that few in vivo model systems exist that closely mimic the natural history of progressive and metastatic human prostate cancer. By means of constructing a cell-cell recombination model [3,4], our laboratory has observed the growth and androgen-independent progression in a human LNCaP prostate cancer model. A series of lineage-related LNCaP cell sublines that reflect the various steps of prostate carcinogenesis and progression has been derived [5,6]. An androgen-independent (AI) cell line, C4-2, reproducibly and consistently follows the metastatic patterns of hormone-refractory prostate cancer by producing lymph node and bone metastases when injected either s.c. or orthotopically in either hormonally intact or castrated hosts [5,6]. This model permits the study of factors that determine the tropism of prostate cancer cells for the skeletal microenvironment.

The goals of the present study are twofold. First, we wish to establish rapid bone metastatic LNCaP human prostate cancer sublines from an animal model of human prostate cancer skeletal metastasis for future mechanistic and therapeutic studies. Second, we wish to characterize these LNCaP sublines with respect to their biologic, cytogenic, and biochemical characteristics. Our findings indicate that: 1) Stromal cells and host endocrine status play a pivotal role in "selecting" or "inducing" prostate cancer cells to acquire androgen-independence and osseous metastatic potential. 2) Specific cytogenetic alterations are associated with LNCaP sublines as a result of androgen-independence and metastatic progression. 3) Anchorage-dependent rather than -independent growth of the LNCaP sublines correlated with prostate cancer invasion in vitro and their metastatic potential in vivo. 4) PSA expression by LNCaP sublines appears to be dysregulated and is independent of the presence of androgen but is highly sensitive to regulation by undefined host factor(s). The LNCaP prostate cancer progression model reveals a basic principle in which epigenetic factor(s) derived from the host can "drive" the progression of a human prostate cancer cell line, LNCaP, to androgen-independence as well as to acquire osseous metastatic potential with defined cytogenetic changes.

MATERIALS AND METHODS

Establishment of an In Vivo Human Prostate Cancer Model

LNCaP cells, passage 29 of the original line developed by Horoszewicz et al. [7], were kindly supplied by Dr. Gary Miller (University of Colorado, Denver, CO). Passages 25–33 of a human bone fibroblast cell line, MS, established from a patient with an osteogenic sarcoma as described previously [8], were used in this study.

Six- to eight-week old athymic nude mice (ncr strain [Balb/c background]), obtained from Charles River Laboratories (Baltimore, MD), were used for all in vivo experiments. They were kept under pathogen-free conditions in laminar flow boxes in accordance with established institutional guidelines and approved protocols. Unless otherwise specified, LNCaP tumors were induced by s.c. coinjection of 1×10^6 LNCaP cells and 1×10^6 MS cells into male athymic nude mice, as described previously [8]. Typically, cells were inoculated s.c. at 2–6 sites into the flank of intact mice. After 8 weeks, some mice were castrated bilaterally by scrotal incision under methoxyflurane

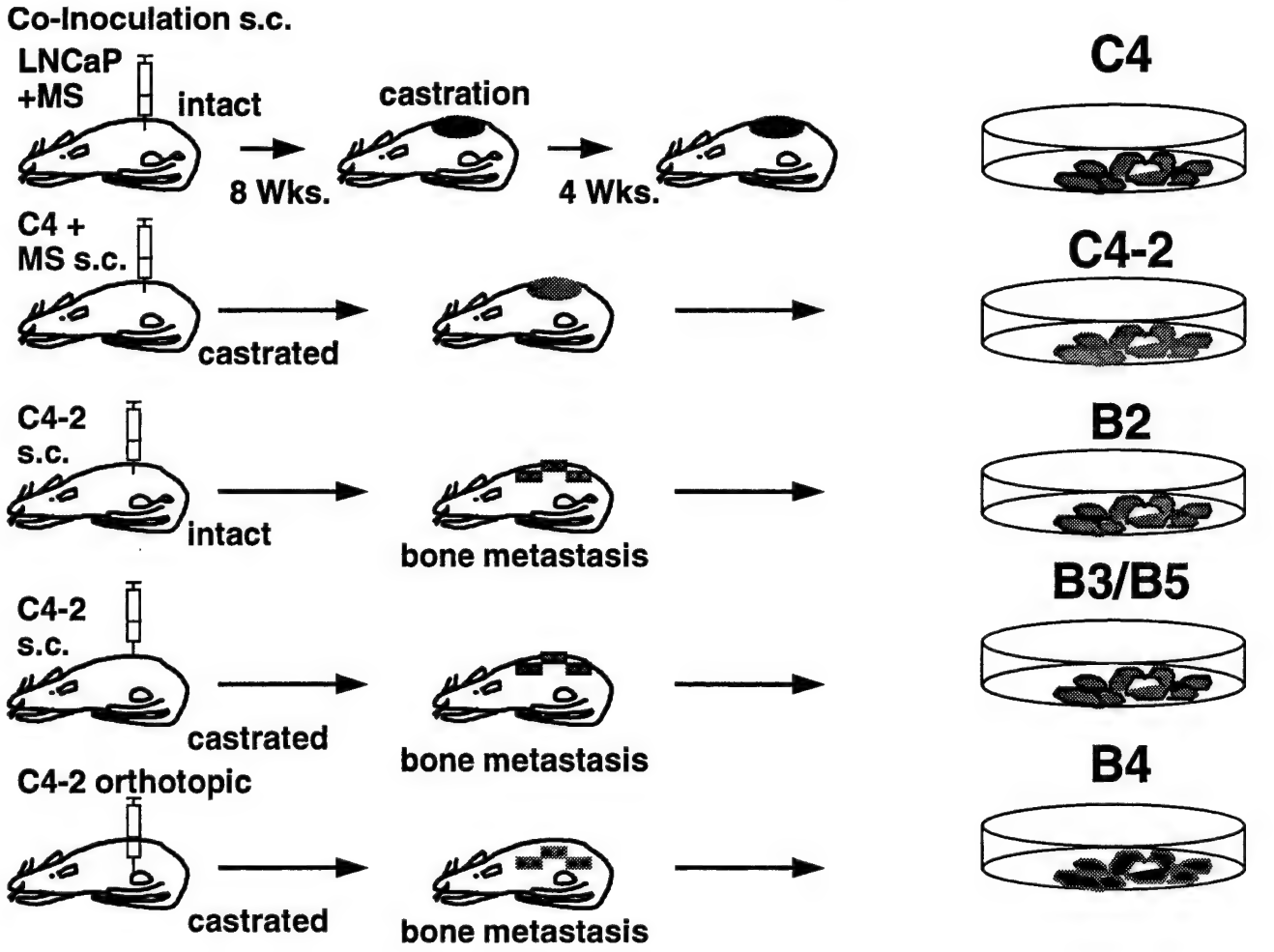

Fig. 1. Schematic derivation of LNCaP sublines from tumors maintained in intact and castrated athymic male mice. MS, fibroblasts derived from a human osteosarcoma.

(Metofane®) anesthesia, and others were sham-operated. The tumors were maintained in the castrated hosts for an additional 4 or 5 weeks (Fig. 1).

The C4 and C5 LNCaP sublines were established from tumors harvested from the castrated hosts at 4 weeks (C4 subline) and 5 weeks (C5 subline) postcastration, as described [8]. A control LNCaP subline (subline M) was established from a LNCaP tumor maintained in an intact male host for 12 weeks (a sham operation was performed at 8 weeks), as described [8]. The C4-2 subline was established in a similar manner from a C4/MS chimeric tumor grown in a castrated male host [5]. All cell lines, passages 23–35, were grown in T-medium as described [9]. The cells were tested and found free of *Mycoplasma*.

For subcutaneous injection of C4-2 cells (1.0×10^6 cells/site), the cells were resuspended in RPMI-1640 and 10% FBS and injected in 0.1 ml/site (27-gauge needle, 1-ml disposable syringe) at 6 sites per mouse. For orthotopic administration of the tumor cells (1.0×10^6 cells), the cells were resuspended in the same medium (total volume of 20 μl) and were delivered to the dorsolateral lobe of the prostate gland of athymic mice by a 30-gauge needle, using a calibrated push-button Hamilton syringe (Reno, NV). Orthotopic injections were performed under methoxyflurane (Metofane®) anesthesia, with the prostate lobe exposed following lower midline abdominal incision. The wound was closed by metal clips (Autoclip, Clay Adams, Parsippany, NJ).

Establishment of Fast-Growing Human Bone Metastatic Cell Lines

In order to study the in vivo behavior of bone metastasis, we derived fast-growing sublines B2–B5 derived from bone metastases that have a defined cell lineage relationship with C4-2 (Fig. 1). C4-2 tumor cells, which adhere more loosely to culture dishes than fibroblasts and osteoblasts, were recovered by me-

chanically irrigating these cells with tissue culture medium. Pure bone metastasis-derived sublines B2–B5, as judged by morphological, cytogenetic, and immunohistochemical criteria, were obtained after 8–12 rounds of these subculturing steps.

The bone metastasis-derived sublines B2–B5 were grown in T-medium (see above) with 5% FBS. The cells were routinely tested and found to be free of *Mycoplasma*. Unless indicated otherwise four mice were injected (27-gauge needle, 1-ml disposable syringe) subcutaneously with 1.0×10^6 B2, B3 (8 mice), B4, and B5 cells resuspended in 0.1 ml of T-medium and 10% FBS at 4 sites per animal. (The mice were bilaterally castrated by scrotal incision under methoxyflurane (Metofane®) anesthesia. The wound was closed by metal clips. Animals were routinely inspected for physical abnormalities and tumor growth. Tumors were measured weekly, and their volumes were calculated by the formula $L \times W \times H \times 0.5236$ [10].

Characterization of the Parental and LNCaP Sublines: Crystal Violet Growth Assay

Cellular growth rates for LNCaP, C4-2, and B2–B5 sublines were determined using a 96-well plate (Becton Dickinson, Lincoln Park, NJ) assay based on the uptake, retention, and elution of crystal violet. Cells were plated at low density (0.5×10^3 cells) in 24-well plates (Becton Dickinson) and incubated for up to 8 days. At days 2, 4, 6, and 8, the cells were fixed with 1% glutaraldehyde (Sigma Chemical Co., St. Louis, MO) and stained with 0.5% crystal violet (Sigma) and washed as described [11]. Crystal violet dye was eluted with 0.5 ml of Sorenson's solution (9 mg of trisodium citrate in 305 ml of distilled H_2O, 195 ml of 0.1 N HCl, and 500 ml of 90% ethanol), and 100 μl were transferred to a 96-well plate. In all experiments the absorbency of each well was measured by a Titertek Multiscan TCC/340 (Flow Laboratories, McLean, VA) at 560 nm, unless otherwise specified. Control experiments demonstrated that absorbance is directly proportional to the number of cells in each well. Experiments were performed in three wells per cell line. Experiments were repeated twice.

Measurement of Prostate-Specific Antigen (PSA)

Once tumors became measurable, blood samples for sequential PSA measurements were obtained by dorsal tail vein incision. Samples were collected in 75-mm microhematocrit capillary tubes and centrifuged, and the sera were stored at −20°C until further processing. PSA values in the serum were determined by a microparticle enzyme immunoassay (MEIA) for the quantitative measurement of PSA in an Abbott IMx clinical analyzer (assays kindly provided by Abbott Laboratories, Abbott Park, IL).

For in vitro PSA measurement, cells were plated on Falcon plastic dishes (Becton Dickinson) and grown in T-medium to 80–100% confluence. After changing of the medium, PSA was measured in the supernatant 24 hr later as described above, and normalized to the number of cells in the dish. Experiments were repeated 3–7 times per cell line.

Cytogenetic Analysis

Cultures from B2–B5, fed 24 hr earlier with fresh medium, were exposed to Colcemid (final concentration, 0.02 μg/ml) for 40 min at 37°C. Cells from these cultures were dislodged by exposing them to 2 ml Hank's balanced salt solution containing 0.01% trypsin. The single-cell suspension in 5 ml of RPMI-1640 containing 10% FBS was centrifuged at 1,700 rpm for 5 min. After discarding the supernatant, the cell pellet was disturbed and exposed to a hypotonic solution (0.06 M KCl) for 15–20 min. at room temperature. After centrifugation (1,700 rpm for 5 min), cells were fixed in acetic acid:methanol (1:3, v/v) for 15 min and then washed three times in the fixative. Conventional air-drying chromosome preparations were made following routine laboratory techniques, as described elsewhere [12].

Invasiveness

To determine the invasiveness of the different cell lines, Costar 12-well plates (Costar, Cambridge, MA) having a polycarbonate filter insert (polyvinylpyrrolidone-free, 8-μm pore size) were coated with commercial Matrigel (Collaborative Biomedical Products, Bedford, MA) at a 1:3 dilution with serum-free T-medium and allowed to gel in a 37°C incubator for 30 min. LNCaP, C4-2, and B2–B5 cells were trypsinized to single-cell suspensions, and 2.5×10^3 cells in 0.5 ml of T-medium (5% FBS) were placed onto the Matrigel. One milliliter of T-medium (5% FBS) was placed in the lower compartment. Assays were incubated for 24 hr at 37°C with 95% air plus 5% CO_2. The filters on the side of the lower compartment were stained with crystal violet, and after washing, the remaining dye was wiped with a cotton swab and the dye on the swab was eluted with Sorensen's solution. Then the cells in the upper compartment were stained, washed, and eluted. The absorbance of each well was measured. Invasiveness was calculated as percent (%) absorbance of the lower compartment/absorbance of the upper and lower compartment. Experiments were performed in triplicate.

Intrinsic Tumorigenic Activity of the Bone Metastatic Cell Lines

Intrinsic anchorage-independent growth (soft agar colony formation) activity in vitro closely reflects the tumorigenicity of target epithelial cells [13]. Therefore, 2.5×10^3 LNCaP, C4-2, and B2–B5 cells were trypsinized to single-cell suspensions; the cells were resuspended in 0.3% agarose containing T-medium supplemented with 2% TCM and plated on a 12-well plate (Costar) containing 0.6% agarose as a bottom layer. The cover layer, consisting of T-medium and 2% TCM, was changed weekly. Colonies larger than 0.4 mm were scored 6 weeks after plating.

RNA Blot Analysis

LNCaP, C4, C4-2, and B2–B5 cells were cultured to 70–80% confluence; 48 hr prior to harvesting, cells were downshifted to serum-free conditions. Each cell line was then treated with 10^{-9} M dihydrotestosterone (DHT) for 48 hr. Cells remaining under serum-free conditions served as control for basal PSA mRNA expression. Total cellular RNA was extracted from cells by the RNAzol B® method (BiotecX Laboratories, Inc., Houston, TX), a single-step purification protocol described by Chomczynski and Sacchi [14]. Equal amounts of RNA as determined by absorbance at 260 nm were subjected to RNA blot analysis by electrophoresis in a 0.9% agarose gel containing 2 M formaldehyde. RNAs were transferred by capillary blotting onto Zetaprobe® membrane (Bio-Rad, Richmond, CA), using 1 × TAE (0.04 M Tris-acetate, 1 mM EDTA) buffer. RNAs were cross-linked to the membranes by ultraviolet exposure, using a Stratalinker® (Stratagene, La Jolla, CA) at 1,500 μJ, and membranes were prehybridized in hybridization buffer (10% dextran sulfate, 1% standard saline citrate, 1 M NaCl, and 20 μg/ml salmon sperm DNA). The solution hybridization was performed by incubation at 65°C overnight by exposing the membranes to a ^{32}P-labeled PSA cDNA probe having a specific activity $>1 \times 10^8$ dpm/μg. After hybridization, the membranes were washed in 2 × standard saline citrate at room temperature for 30 min, and were then washed under highly stringent conditions (2 × 30 min in 2 × standard saline citrate/1% sodium dodecylsulfate, then 1 × 30 min 0.5 × standard saline citrate/1% sodium dodecylsulfate) at 65°C. Autoradiograms were prepared by exposing Kodak X-Omat AR films to the membrane at −80°C with intensifying screens. Autoradiograms of RNA blot analysis for PSA were analyzed and PSA values were normalized to 18 S mRNA by means of ImageQuant™ (Molecular Dynamics, Sunnyvale, CA).

Histomorphologic Characterization of Tumors

Specimens for routine histological examination were fixed in 4% paraformaldehyde and 5 mM $MgCl_2$. Six-micron paraffin-embedded tumor sections were cut and stained with hematoxylin and eosin. Bone metastases were decalcified in 0.25 M EDTA.

RESULTS

In a previous study [6], we established an androgen-independent, tumorigenic, and bone metastatic cell line, C4-2, through a series of coinoculations of the human prostate cancer cell line LNCaP and the bone stromal cell line MS. In the present study, we focused on the characterization of the LNCaP cell sublines that metastasize to the bone and cell lines derived directly from those bone metastases. As shown in Figure 1, the AI C4-2 subline and the four AI bone metastasis-derived sublines, denoted as B2–B5, were derived from C4-2 tumors grown repeatedly in castrated male hosts, with the exception of B2, which was grown in an intact male host.

Cytogenetic analysis demonstrated that all four C4-2 bone metastasis-derived sublines (B2–B5) were of human origin [5,6]. The chromosome numbers in these C4-2 sublines varied between 80–89. Figure 2A–E shows typical G-banded karyotypes of the parental C4-2 and its bone metastasis-derived sublines, B2–B5. The cell lineage relationship was confirmed by common marker chromosomes, which were chromosomes 1, 2, 6, 10, 13, 15, and 16. Chromosome 8 had an 8p deletion in all sublines. B4 and B5 did not acquire any new marker chromosomes. The B2 subline acquired both stochastic deletion of the Y chromosome and the appearance of a new marker, a translocation between chromosomes 3q and 12q. B3 acquired two marker chromosomes: m1 = i(7q) in some metaphases, and m2 = der (11)t(11p+?). These markers appear to be associated with metastatic LNCaP sublines [5,6]. B5 is deleted for Y chromosome.

The growth curve of the parental LNCaP cell line in vitro was distinctly slower than that of its sublines C4-2, B2, B3, B4, and B5, as shown in Figure 3A. Previous studies showed that soft agarose colony-forming activity correlated well with in vivo tumorigenicity [3]. Results of this study, however, showed that anchorage-independent growth of C4-2 and B4, but not B2, B3, and B5 sublines, reflected accurately the in vivo growth potential of these bone metastasis-derived cell lines (Fig. 3B, Table I). Although there appear to be correlations between the latency of tumor growth, with B4 tumors growing faster than B2, B3, and B5, the frequencies of anchorage-independent

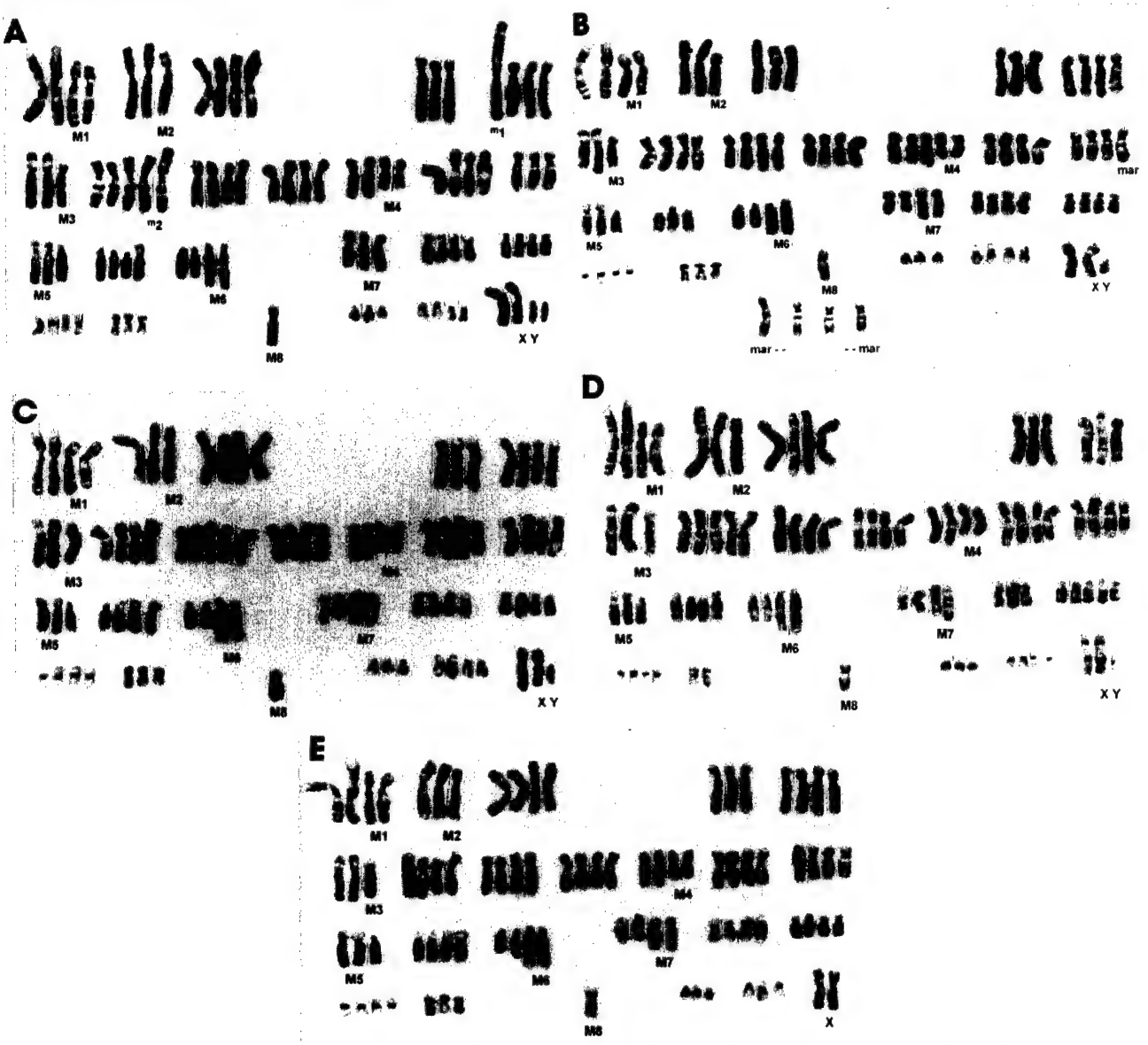

Fig. 2. Cytogenetic analysis: representative G-banded karyotypes for each of the cell lines, C4-2 (**A**), and C4-2 B2–C4-2 B5 (**B–E**), are displayed. Karyotype analysis confirms that cell lines are both human and derived from the C4-2 parental cell line, which was used to derive the bone metastatic variants.

growth of the latter were substantially lower than those of the parental LNCaP cells, which were shown to be nontumorigenic and nonmetastatic when inoculated subcutaneously in athymic hosts [3,5,6]. In comparison to the parental LNCaP cell line, all of the metastatic LNCaP cell sublines (C4-2, and B2–B5) presented a 2–3-fold higher invasiveness in a transwell Matrigel invasion assay (Fig. 3C).

RNA blot analysis indicates that all LNCaP sublines with a defined cell lineage relationship express mRNA for prostate-specific antigen (Fig. 4A,B). PSA mRNA expression can still be induced by dihydrotestosterone (DHT) at physiologic concentrations, but to a lesser degree in the AI cell lines (Fig. 4A,B). Interestingly, androgen slightly suppressed PSA expression in the B3 cell line (Fig. 4B). Due to the unexpectedly low level of PSA mRNA in the B4 subline, we subcloned this line to determine whether this was clonal variation among B4 clones. Several single-cell clones of B4 (such as B4 (6-1); see Fig. 4A,B) were observed to have elevated PSA mRNA levels. Conversely, an additional observation was made when reinjecting the B3 cell line in vivo. A cell line isolated from a B3 tumor expressed high levels of PSA in vivo, but upon subcloning of a

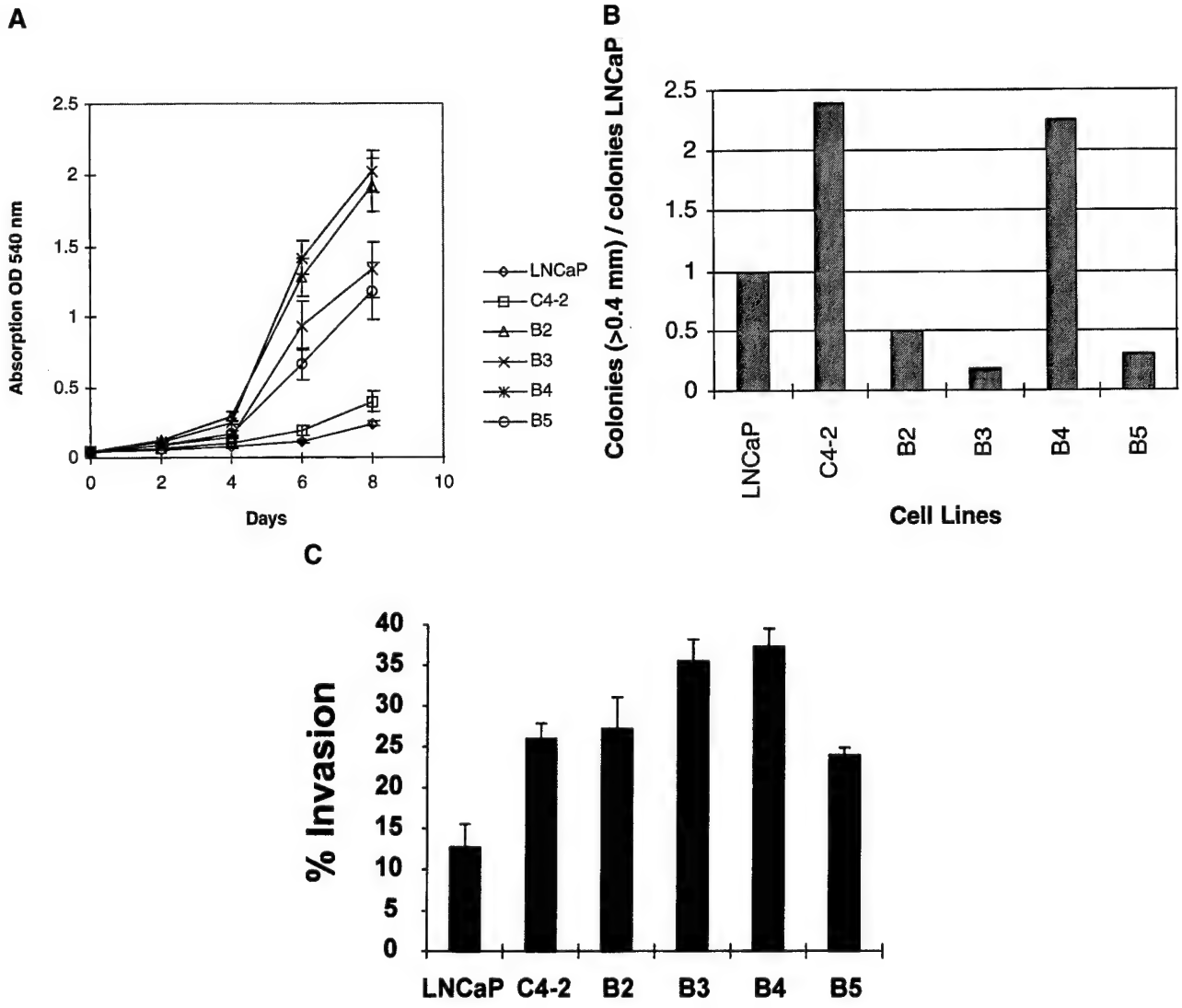

Fig. 3. In vitro assays. **A:** In vitro growth curves in 5% FCS assessed in a crystal violet growth assay. **B:** Anchorage-independent growth in a soft agar colony formation assay, normalized to LNCaP. **C:** Invasiveness of LNCaP and sublines in a Matrigel invasion assay expressed in percent of cells invading Matrigel.

TABLE I. Tumor Formation and Metastasis in Castrated Host by Subcutaneous Injection

Cell line	Tumor formation s.c.	Latency (median; range)	Paraplegia	Latency (median; range)
B2	4/4 (100%)	11 wks (10–12)	1/4 (25%)	19 wks (19)
B3	7/8 (87.5%)	11.2 wks (11–13)	3/8 (37.5%)	14.3 wks (13–16)
B4	4/4 (100%)	7.3 wks (5–11)	1/4 (25%)	5 wks (5)
B5	4/4 (100%)	10.7 wks (9–12)	1/4 (25%)	16 wks (16)

B3 tumor, the B3.1 subclone expressed substantially lower levels of PSA in vitro (Fig. 4A,B). These results suggest that the microenvironment of the tumor cells plays a key role in determining the steady-state levels of PSA expression in prostate tumor cells.

RNA blot analysis for the human androgen receptor consistently demonstrated a 9.4-kb transcript for the human androgen receptor in all cell lines (data not shown).

To determine if the C4-2 sublines were metastatic in vivo, we compared both the tumorigenicity and the

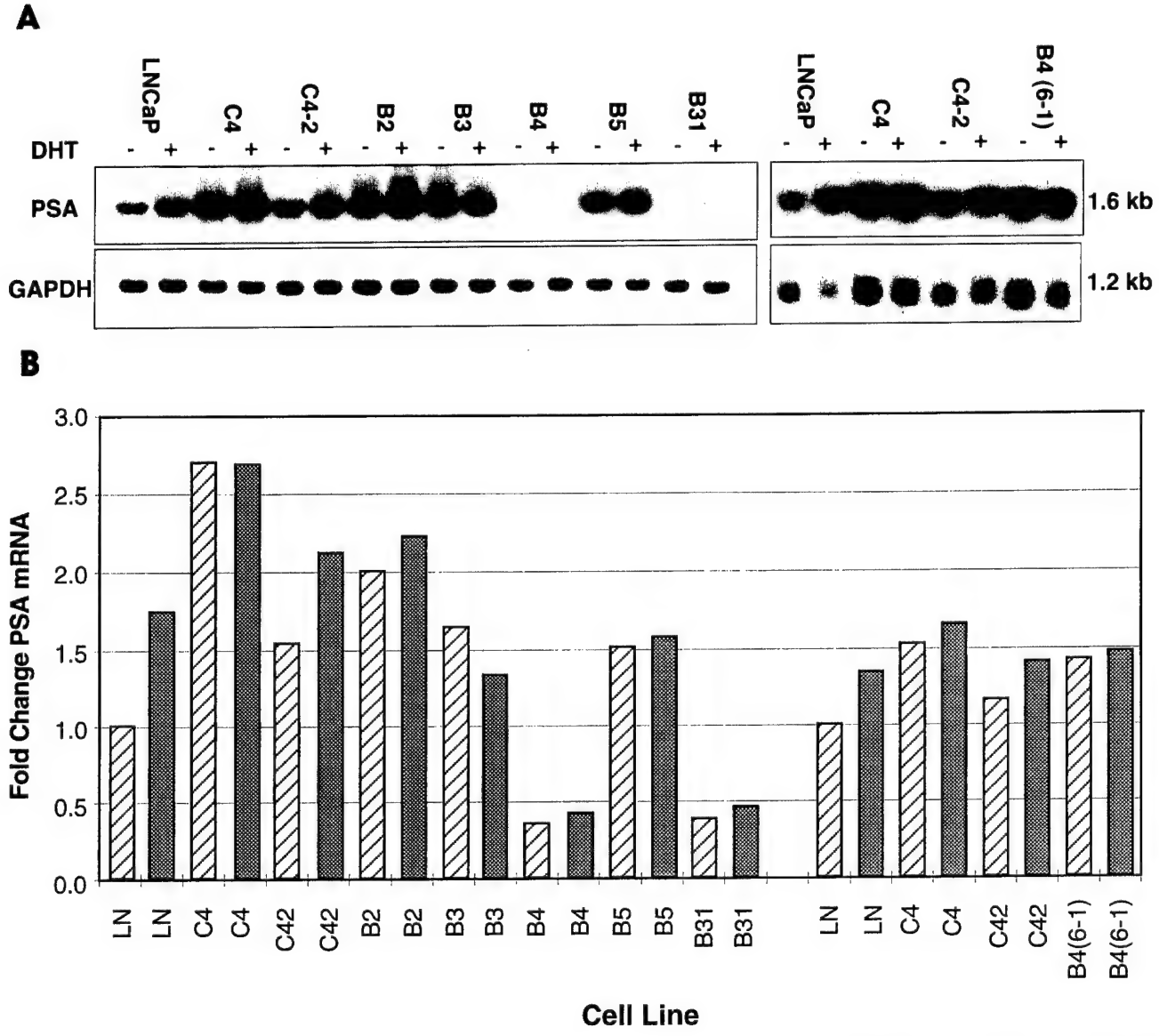

Fig. 4. Regulation of PSA expression in the LNCaP cell lineage model by dihydrotestosterone. Cells were exposed to dihydrotestosterone (DHT) at 10^{-9}M for 48 hr under serum-free culture conditions. **A, left:** Androgen-induced PSA mRNA expression in parental LNCaP and its lineage-derived sublines. Intrinsic PSA was lower in the B4-derived subline from an early passage culture. DHT induced an increased PSA mRNA in the androgen-dependent rather than the androgen-independent LNCaP sublines. Androgen-independent C4-2 and its derivative bone sublines responded to a lesser extent than the parental LNCaP to DHT. **Right:** Responsiveness of PSA mRNA to DHT in the LNCaP subline, B4 variant 6-1. Note that this variant has a much higher basal level expression of PSA; it remained unresponsive to DHT. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as loading control. **B:** Determination of the fold change in PSA mRNA expression with (+) and without (−) treatment with DHT. All data are expressed as fold change PSA/GAPDH and are normalized to LNCaP without DHT treatment.

metastatic potential of these C4-2 sublines. Whereas the incidence of tumor formation in mice inoculated with 1.0×10^6 cells of the LNCaP subline C4-2 was affected substantially by the site of injection (100% orthotopic vs. 1.5% s.c.) [6], the tumorigenicity of 1.0×10^6 cells each of the four bone metastasis-derived sublines, denoted B2–B5, was not influenced by the site of injection; subcutaneous reinoculation of the bone metastasis-derived cell lines B2–B5 resulted in rapid tumor growth, with incidences ranging from 87.5–100% (Table I). Bone metastasis-derived cell lines grew tumors more rapidly in vivo than the C4-2 cell line (5–13 weeks as compared to 9–14 weeks), and a fraction of the animals (25–37.5%) developed paraplegia faster

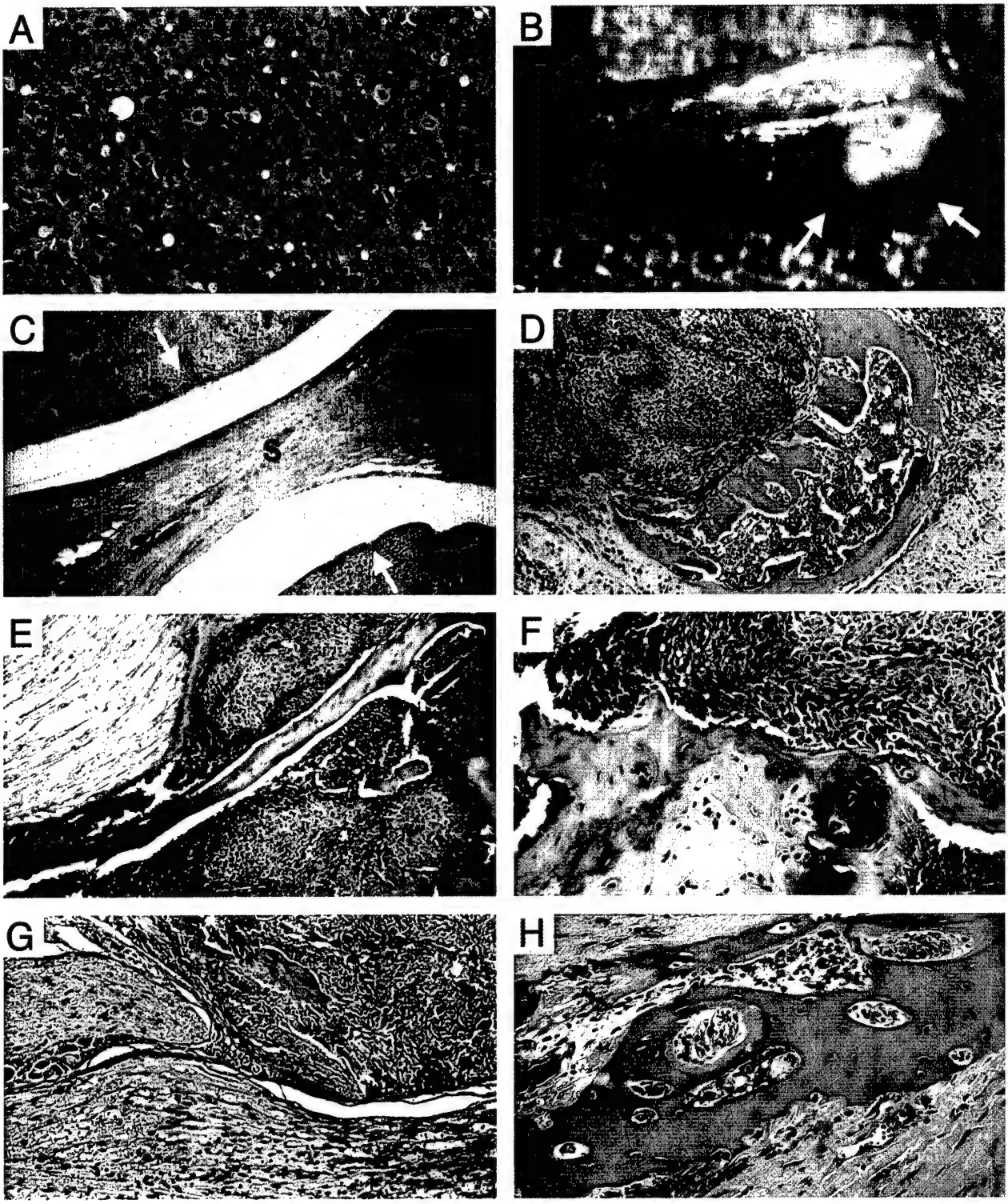

Fig. 5. Macroscopic and histological characterization of LNCaP sublines. **A:** Primary tumor of the B4 subline. Note the presence of multiple mitotic figures. **B:** Dissected spine of a paraplegic animal due to osseous metastasis of the B4 subline. Arrows indicate enlarged spinal metastasis. **C:** Longitudinal section of a spine with osseous metastasis and compression of the spinal cord. Arrows indicate the encroaching bone metastasis resulting in spinal compression. **D:** Spinal metastasis resulting from the B2 subline. Note the poor organization and high degree of dysplasia found in this tumor. **E:** Spinal metastasis derived from a B3 subline. Note a poorly organized carcinoma encroaching on the spinal cord. **F:** High magnification of a B3-derived spinal metastasis. This high-magnification view clearly shows osteoblastic response and bone remodeling by the B3 subline. **G:** Low-power view of a B4 spinal metastasis. Note poorly organized carcinoma with remodeled and newly deposited osteoid encroaching upon the spinal cord. **H:** High-power view, showing bone remodeling from a B5 subline-derived spinal metastasis. Newly deposited osteoid contains carcinomatous elements (A, F, H, ×62.5; C, D, E, G, ×31.25).

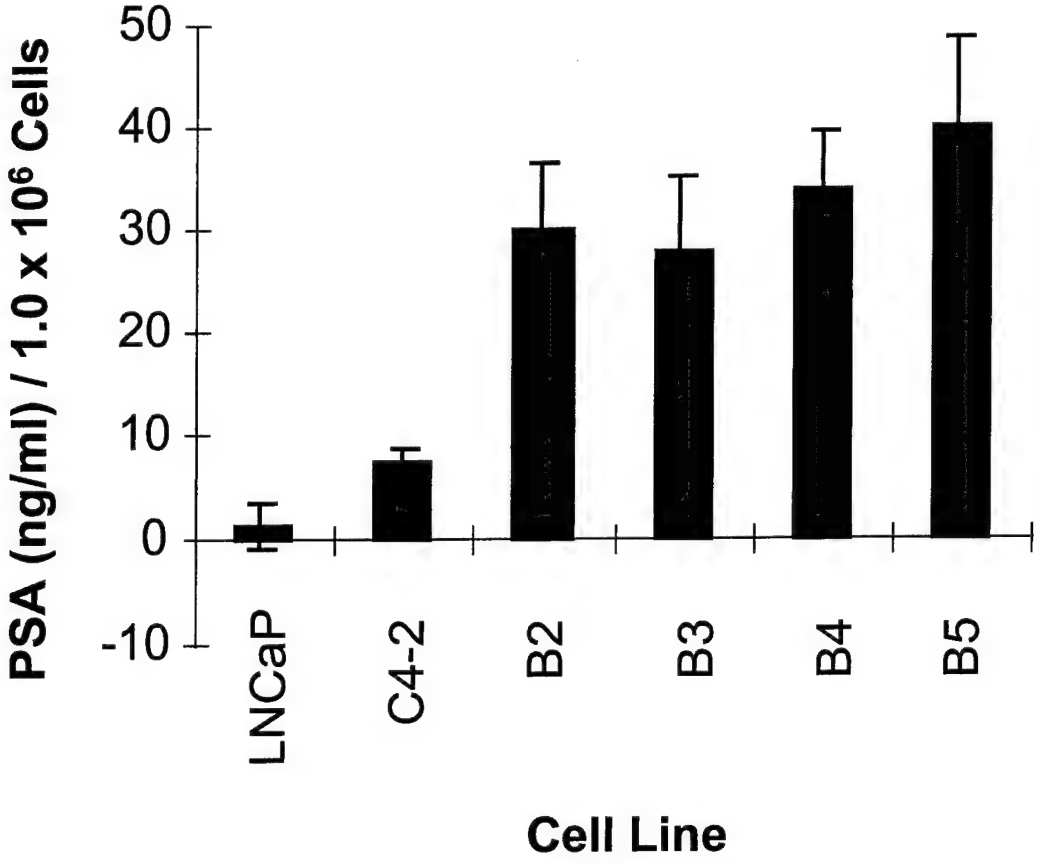

Fig. 6. Secretion of PSA protein measured in ng/ml of LNCaP and lineage-derived sublines in vitro. Data were expressed as mean (± standard deviation) PSA protein secreted by the different cell lines per 1.0 x 10^6 cells.

(5–19 weeks) than those animals inoculated with C4-2 cells (mean, 29 weeks; range, 17–43 weeks; see Thalmann et al. [6]). Histomorphologically, the primary tumors were poorly differentiated (Fig. 5A).

One animal inoculated subcutaneously with B4 cells developed osseous metastasis as early as 5 weeks. Overall, the bone metastasis-derived cell lines had a bone metastasis incidence of 33.3% (range, 25–37.5%). Seven of 20 (35%) animals developed cachexia prior to osseous metastasis and had to be sacrificed.

Bone metastases (Fig. 5B) induced a vast osteoblastic reaction (Fig. 5D–H), with compression of the spinal cord and subsequent paraplegia (Fig. 5C). Figure 5D–H depicts a panel of osseous metastases (B2–B5). The osteoblastic tumor deposits had an increased basal metabolism, and hence elevated uptake and sequestration of 99mTechnetium-methylene diphosphonate in whole-body sagittal imaging bone scans, as shown previously [6] (data not shown).

PSA expression was generally high initially in animals carrying LNCaP subline-derived tumors, but PSA became greatly depressed when tumors were maintained in hosts for longer periods (>5 months, data not shown). Animals injected with C4-2 or bone metastasis-derived LNCaP sublines developing osseous metastases and paraplegia tended to secrete lower levels of PSA in vivo than animals who did not develop bone lesions. Interestingly, this was reversed in vitro. As demonstrated in Figure 6, the bone metastasis-derived cell lines secreted up to 20-fold more PSA per million cells into the medium than the parental LNCaP cell line. The B4 cell line expressed large amounts of PSA protein in vitro, despite low mRNA levels.

The phenotypic characteristics of the LNCaP model of prostate cancer progression is summarized in Table II and indicates that by passaging cells through hosts, the phenotype can be altered permanently in terms of tumorigenicity, metastatic potential, cytogenetic status, and biologic behavior.

DISCUSSION

The process of metastasis is a cascade of linked sequential steps involving complex genetic and epigenetic interaction [4,15]. To gain further insight into the molecular events that lead to cancer metastasis and to establish useful experimental models of prostate can-

TABLE II. Phenotypic Characterization of Parental LNCaP, Androgen-Independent Sublines C4 and C4-2, and Bone Metastasis-Derived Cell Lines B2, B3, B4, and B5

Cell line	No. of in vivo passages	Androgen-dependent	PSA production	Tumorigenicity		Metastasis to the skeleton
				Male	Castrated host	
LNCaP	0	Yes	Yes	No	No	No
C4	1	No	Yes	Yes	No	No
C4-2	2	No	Yes	Yes	Yes	Yes
B2	3	No	Yes	Yes	Yes	Yes
B3	3	No	Yes	Yes	Yes	Yes
B4	3	No	Yes	Yes	Yes	Yes
B5	3	No	Yes	Yes	Yes	Yes

cer metastasis, a number of approaches have been implemented. For example, genetic manipulation of prostate epithelial cells either through oncogene transfection [16–18] or transgenic mice using tissue-specific promoters [19–21] has resulted in low or no incidence of bone metastasis, with little evidence of osteoblastic reaction. Recognizing the importance of the tumor-host microenvironment interaction in dictating tumor behavior in vivo [4,22,23], we have derived human prostate cancer cell lines from tumor xenografts or their metastases by subculturing tumor cells from chimeric xenografts [3–6,8], and rat prostate cancer epithelial and stromal cell lines by altering the host microenvironment [9]. These efforts resulted in obtaining prostate tumor cells with defined tumorigenic and metastatic potential.

In the development of an in vivo mouse model of human prostate cancer metastasis, we observed that a marginally tumorigenic human prostate cancer cell line, LNCaP, can be induced to acquire and/or adapt to these changes, resulting in AI progression, with the resulting cell acquiring tumorigenic and osseous metastatic phenotypes when grown under androgen-depleted conditions with inductive prostate or bone stromal cell lines in vivo [5,6,8]. These findings exemplify the key role of stromal cells and the hormonal status of the host in directing cell-cell interaction and dictating the changes leading to the subsequent expression of androgen-independent and metastatic phenotypes. The present communication documents the genotypic and phenotypic alterations of human prostate cancer cell lines derived from spontaneous experimental bone metastases of LNCaP lineage-derived cell sublines. These LNCaP sublines have acquired diverse tumorigenic and metastatic potential.

The LNCaP model of prostate cancer progression mimics the clinical features of human disease: 1) Androgen-dependent (defined as prostate tumor or cells capable of growing subcutaneously and secreting PSA only in intact male hosts when inoculated with supporting stromal cells or extracellular matrix) LNCaP cells progress to become androgen-independent (defined as cells capable of forming PSA-secreting prostate tumors in castrated male hosts without supporting stromal cells or extracellular matrix) upon interaction with bone stromal cells in castrated hosts, resulting in the derivation of lineage-related LNCaP sublines C4-2, and B2–B5. 2) Androgen-independent LNCaP sublines synthesize and secrete PSA in an AI manner. However, a reduced level of PSA expression by androgen-induction was observed. The LNCaP derivative sublines express androgen receptor, and this receptor is functional and can be stimulated by physiologic and superphysiologic concentrations of exogenous androgens. 3) When grown orthotopically, LNCaP sublines induced localized prostate cancer and growth, invading the seminal vesicles and metastasizing to the lymph nodes and the bone, where they invoked a marked osteoblastic reaction of the skeleton. Animals subsequently developed signs of paraplegia and cachexia followed by death [6]. The behavioral patterns of invasion, migration, and dissemination are commonly observed in men with clinical prostate cancer. 4) Analysis by means of comparative genomic hybridization (CGH) [24] confirmed the cell-lineage relationship between the parental LNCaP and its sublines. Although the regions of chromosomal losses or gains are not in complete concordance using either CGH or conventional cytogenetics, it was noted that marked chromosomal changes occurred in LNCaP bone metastasis-derived sublines, as defined by both conventional (Fig. 2) and CGH [24] techniques. Remarkably, 15 of the 16 genetic changes documented in the C4-2 human prostate cancer progression cell line [24] were reported in cytogenetic studies of clinical specimens of human prostate cancer [25–30]. These results suggest that the LNCaP human prostate cancer progression model has the unique advantage of

closely mimicking the phenotypic and genotypic changes often found in clinical human prostate cancer.

PSA mRNA expression is elevated in the LNCaP sublines (Fig. 4A,B). The fold of androgen induction of PSA mRNA decreases with the acquisition of androgen-independence (Fig. 4B). Interestingly, the B4 cell line, which expresses PSA on the protein level in vitro and in vivo, expressed low amounts of PSA mRNA in vitro. Single B4 cell clones were then expanded and showed PSA expression comparable to that of the other bone metastasis-derived cell lines, indicating clonal diversity. This is also supported by the fact that a B3-derived subline, expressing high PSA in vivo, demonstrated low levels of PSA mRNA expression in the derivative B3.1 subline (Fig. 4A,B). In other cases, however, PSA was expressed initially in the tumor xenografts (e.g., C4-2 tumors), but the levels of PSA decreased markedly as tumors were maintained in the castrated hosts for longer periods until bone metastasis was detected. We attribute this to downregulation of PSA expression by soluble factors secreted by bone stromal cells. Koeneman et al. (unpublished observations) showed that PSA promoter-reporter expression in LNCaP and C4-2 cells is depressed by the conditioned media obtained from bone stromal cell lines. Finally, all LNCaP sublines expressed PSA at the protein level, with the level of PSA expression positively correlated with the osseous metastatic potential of the derivative sublines (Fig. 6).

Of the four bone metastasis-derived cell lines, only B4, which was derived from a castrated host that was injected with C4-2 cells orthotopically (presumably cellular interaction occurs between C4-2 cells and the host mouse prostate stroma), showed increased tumorigenicity and rapid development of bone metastases when subsequently injected s.c. in athymic castrated male mice. The other bone metastasis-derived C4-2 sublines (B2, B3, and B5) originated from subcutaneous administration of C4-2 cells in intact or castrated hosts. These observations suggest that the stromal-epithelial interaction (with stromal cells derived either from the skin or the prostate) under androgen-depleted conditions might subselect cell clones with a more malignant phenotype. A growing body of experimental evidence supports the concept that androgen deprivation enhances the development of androgen-independent growth and the acquisition of metastatic potential by prostate cancer cells. In the Noble [31] and Dunning [32] rat models, androgen-independent and metastatic prostate cancer cell clones were selected from tumors maintained in androgen-deprived male hosts. In the androgen-dependent Shionogi mouse mammary tumor model [33], castration produced an androgen-independent subclone. In the present LNCaP progression model of human prostate cancer, we have demonstrated that such selection/adaptation may occur in tumors maintained in castrated hosts. These results may have profound implications when considering the natural course of prostate cancer androgen-independent progression in androgen-deprived hosts. After hormonal therapy, prostate cancer frequently progresses to an androgen-refractory state. A subset of these patients with androgen-independent disease, treated with maximal androgen blockade using nonsteroidal antiandrogens, may progress and could benefit from a withdrawal of the nonsteroidal antiandrogens such as flutamide and bicalutamide [34]. This may be a consequence of prolonged androgen-deprivation. Based on results obtained from tissue culture and animal models, it was suggested that long-term culture of prostate cancer cells in an androgen-deprived environment [35,36] results in the development of androgen suprasensitivity or androgen receptor mutations. It is conceivable that intermittent androgen suppression, which is currently being evaluated in clinical trials, might depress the emergence of androgen-independent clones from the stem-cell populations within prostate tumors [37].

Overall, these findings illustrate the importance of the host and tumor environment on the biologic behavior of tumor cells. Although vascular anatomy [38,39] may account in part for the selectivity of prostate cancer for the axial skeleton, considerable experimental evidence [3,5,6,8,13,40] has substantiated that local factors, such as bone fibroblasts, extracellular matrix, and growth factors, play an important role in tumor progression and metastasis, thus supporting Paget's "seed in fertile soil" theory [41].

In summary, we established an osseous metastatic model of human prostate cancer. Evidence suggests that androgen-deprivation may facilitate androgen-independent progression and acquisition of metastatic potential by prostate cancer cells in a nonrandom manner. We have characterized the molecular, biochemical, and cytogenetic characteristics of osseous metastasis-derived LNCaP sublines in vitro and have correlated these findings with the tumorigenicity and metastatic potential of these cells in vivo. This model of human prostate cancer progression and metastasis closely mimics the genetic and pathological processes of cancer growth and progression in men.

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Prostate Carcinoma-Stroma Interaction: Molecular Mechanisms and Opportunities for Therapeutic Targeting[1]

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Abstract

Maintenance of cell and tissue homeostasis is dependent upon the dynamic balance of cell proliferation, differentiation and apoptosis through interactions between cells and between cells and their microenvironment. The unique prostatic cellular phenotypes are induced and maintained by interaction between epithelium and adjacent stroma through intimate intercellular signaling pathways. In this article, we summarize current advances in the carcinoma-stroma interaction and its biologic and therapeutic implications. We specifically emphasize current studies of the possible factors driving the "vicious cycle" between stroma and emerging prostate carcinoma cells that may be responsible for carcinogenesis and metastasis to bone. Stroma responds both genotypically and phenotypically to tumor epithelium upon co-culture under 3-D conditions. Likewise, the emerging carcinoma responds to stromal signals that drive progression to malignancy. A vicious cycle mediated by soluble and insoluble molecules secreted by carcinoma cells and stroma appear be the critical factors supporting and sustaining tumor colonization in bone. Co-targeting tumor and stroma with therapeutic agents has yielded promising results both in pre-clinical models of prostate cancer and bony metastasis and in clinical trials of patients treated with a dual carcinoma and stroma targeting strategies. In conclusion, understanding and targeting the interaction of the carcinoma and its stromal microenvironmant may improve the prognosis, reduce the suffering and increase the survival of patients with advanced cancer metastasis.

Keywords: prostate cancer, bone metastasis, stromal-epithelial interaction, molecular targeting, vicious cycle, cytokine, extracellular matrix (ECM)

Introduction

This review will first elucidate the molecular mechanisms underlying prostate carcinoma-stroma interaction, involving prostatic stromal cells at the primary site and osteoblasts and osteoclasts at bone metastasis sites, and then discuss new opportunities for therapeutic targeting of localized and disseminated human prostate cancer.

Cell and tissue homeostasis reflects a dynamic balance of cell proliferation, differentiation and apoptosis (Frisch and Screaton, 2001). Consistent with this concept, primary and immortalized non-transformed human prostate epithelial cells require adhesion to an extracellular matrix (ECM) to maintain their polarity, growth, survival, and migratory characteristics and expression of tissue-specific proteins. These properties are unique organ-specific phenotypes conferred and maintained by interaction between epithelium and adjacent ECM secreted primarily by the stroma through intimate intercellular signaling pathways. Epithelial cells, the major target for adult cancer, exist in contiguous sheets composed of organized, polarized cells circumscribed by a basement membrane that separates the epithelium from the stroma. Numerous cell types are found in the epithelial and stromal compartments, including luminal, basal and neuroendocrine cells in the epithelial tissue compartment and smooth muscle, fibroblast, endothelial, neuroendocrine, neural and inflammatory cells in the stromal tissue compartment. Intercellular interaction between these cell types, mediated by soluble factors and insoluble ECM, will determine the growth and differentiation potentials of the entire organ.

Homeostasis of normal organs such as prostate and breast is maintained through reciprocal interactions between epithelial cells and their surrounding stroma with minimal proliferation of either cell type. Disruption of the homeostatic interaction between epithelium and stroma could initiate and promote carcinogenesis. In these instances, carcinogenic insults may trigger additional genetic changes in the epithelial cell compartment over and beyond the inherited traits, through increased genomic instability and decreased DNA repair and apoptotic signaling. Altered epithelial cells may trigger stromal reactions that in turn confer reciprocal signal cascades in tumor epithelium to promote further carcinogenic processes. Ultimately, reciprocal carcinoma-stroma interaction culminates in the increased migratory, invasive, and metastatic behavior of cancer cells.

Prostate Carcinoma-stroma Interaction

Prostate epithelium and stroma are sites for the development of benign and malignant diseases of the prostate. Recent evidence suggests that prostate epithelium and stroma interact in a highly organ-specific, androgen-dependent and temporally-related manner. We discuss below the role of prostate fibromuscular cells in prostate tumor growth and progression, the reciprocal stromal reactions to prostate tumor epithelium that create a "vicious cycle" between stroma and epithelium ultimately driving tumor epithelium to develop benign and malignant prostate diseases, the potential factors that may mediate these interactions, and the role of early prostate inflammatory atrophy in the development of benign prostatic hyperplasia and prostate cancer.

Role of prostate fibromuscular stromal cells in prostate tumor growth and progression

In 1970, Professor L. M. Frank described the requirement of prostate cancer fibromuscular

stromal cells for the growth and survival of primary human prostate epithelial cells in culture. We tested the significance of this original in vitro observation in our laboratory by both in vitro cell co-culture and in vivo co-inoculation of tumor cells and stromal cells in immune-compromised mouse models for the growth of human and rat prostatic tumors as xenografts. A series of reports demonstrated that the growth of benign and cancerous prostate epithelial cells in vivo was enhanced markedly by the co-presence of organ-specific and/or cancer-associated stromal cells (Camps et al., 1990; Chung et al., 1989; Gleave et al., 1991; Gleave et al., 1992). These studies were confirmed by other laboratories which showed that prostate tumor growth in vivo could be accelerated by cancer, but not by benign tissue-associated stromal fibroblasts (Olumi et al., 1998; Olumi et al., 1999; Wong and Wang, 2000).

Further, the androgen-independent and metastatic progression of human prostate epithelial cells can be promoted by co-inoculating a marginally tumorigenic human prostate cell line, LNCaP, with a human bone stromal cell line derived from an osteosarcoma in vivo (Thalmann et al., 1994; Thalmann et al., 2000; Wu et al., 1994; Wu et al., 1998). By a series of manipulations of chimeric LNCaP tumor growth in vivo under the influence of bone stromal cells, either in the presence or absence of androgen, the derivative LNCaP sublines C4-2 and C4-2B acquired the ability to become androgen-independent and metastatic, as exhibited by their behaviors in immune compromised mice. To ascertain that cell-cell contact rather than unknown factors from the host were responsible for conferring tumorigenic and metastatic potential to the parental LNCaP cells, we co-cultured LNCaP cells with either prostate or bone stromal cells under 3-dimensional (3-D) conditions and observed similar permanent phenotypic, genotypic and behavioral changes of the parental LNCaP cells, as revealed by their ability to form tumors in castrated mice and acquired ability to metastasize to distant organs including bone (Ozen et al., 1997; Pathak et al., 1997; Rhee

et al., 2001).

These results taken together suggest that tumor stroma can confer "inductive" or "adaptive" cues to the responding tumor epithelium and is directly responsible for the altered behavior of tumor epithelium. However, carcinoma-stroma interaction is reciprocal. Not only can stroma "induce" or "select" the phenotypic and genotypic changes in tumor epithelial cells, tumor epithelium can also induce genetic and phenotypic changes in stroma after tight association in vivo. It appears that ***both*** tumor and stroma are involved in controlling tumor growth or " take" by the host and the subsequent progression of tumor epithelium to androgen-independence and acquisition of local invasive and distant metastatic potential in experimental models of human prostate cancer. Figure 1 depicts a model of the multi-step nature of this interaction. Genetically and phenotypically altered epithelial cells induce a stromal reaction that in turn induces a reciprocal epithelial reaction. The serial interactions form a "vicious cycle" that drives epithelial cancer progressive to androgen-independent local invasion and distant metastasis.

Through growth factor activation, changes in ECM can be elicited, which can cause epithelial cells to lose their apical-basal polarity and thus assume a less well differentiated state (Bissell and Radisky, 2001). This dramatic alteration of epithelial cell phenotype can lead to increased cell proliferation and tumorigenesis (Naishiro et al., 2001; Reichmann, 1994).

However, the specific molecules responsible for tumor-induced changes in the microenvironment and the reciprocal modifications of the tumor by its microenvironment are largely unknown, as are the inter- and intra-cellular pathways that result from these influences. Dissecting the components of the stroma requires model systems in which a single variable can be manipulated and assessed. In contrast to tumor-associated stroma, normal stromal cells have a low proliferative index, probably secrete only the factors necessary to maintain normal tissue function

(Manabe and Owens, 2001), and appear to be less responsive to inductive cues elaborated from normal epithelium.

Stromal reaction to tumor epithelium

Since stromal cells from normal adult tissues are less inductive, or often non-inductive, the experimental data imply that stromal cells exposed to tumor epithelium could be "activated" and acquire an inductive potential to drive the subsequent neoplastic processes. This suggestion is supported by several lines of evidence.

First, morphologic "desmoplastic" stromal response to tumor epithelium often occurs around either primary or metastatic tumor epithelium (Nemeth et al., 1999; Thompson et al., 1993; Tuxhorn et al., 2001). A desmoplastic stromal response is characterized by increased proliferation of fibromuscular stromal cells and enhanced deposition of extracellular matrices (ECMs) surrounding tumor epithelium. This active process could be viewed as a part of the host-defense mechanism to curtail or restrict tumor expansion. Conversely, this reaction and accompanying increased stromal cell number could provide a "fertile soil" supporting the growth and invasion of tumor epithelium through the increased production by stromal cells of growth factors and stroma-associated ECMs. In addition, stromal cells are the major production sites of metalloproteinases, which increase ECM turnover and are thought to be critical to the invasive property of tumor epithelium. Thus, global changes in the tumor microenvironment could provide selective growth and survival advantages for certain tumor cell clones, particularly in androgen-deprived conditions.

Second, stromal reaction to tumor epithelium may be *irreversible*, if the reacting stromal cells receive an "inductive cue" from tumor epithelium to undergo trans-differentiation, whereby

stromal fibroblasts adjacent to tumor epithelium convert both morphologically and phenotypically to myofibroblasts (Elenbaas and Weinberg, 2001; Ronnov-Jessen et al., 1995; Tuxhorn et al., 2001).

Transition of stromal fibroblasts to myofibroblasts with increased expression of vimentin pro-collagen type-I and tenascin has been observed (Tuxhorn et al., 2001). Using the laser capture microdissection (LCM) technique, genetic aberrations were detected in the fibromuscular stromal compartment surrounding tumor epithelium, further supporting the reciprocal nature of the carcinoma-stroma interaction (Moinfar et al., 2000). Although the mechanisms of the genetic and phenotypic response of stroma to adjacent tumor epithelium are currently unclear, possible mechanisms include: a) Transition or inter-conversion of epithelium to stroma (Wernert et al., 2001); b) Irreversible induction of stromal changes, both at the morphologic and the biochemical levels, by soluble and insoluble factors secreted by tumor epithelium; c) Selection of previous existing clones of stromal cell populations and preferential expansion of these clones based on their proliferative and survival advantages (Singer et al., 1995; Tso et al., 2000); d) The combination of b and c above; that is, after prolonged "adaptation" to a tumor-associated stromal microenvironment, permanent genetic changes may occur in the stromal cell population through a poorly understood "adaptive mutation" mechanism (Chung, 1995).

A "vicious cycle" between prostate stroma and tumor may be responsible for carcinogenesis in primary prostate cancer

Studies using tissue and cell recombination models demonstrate that growth and differentiation of the prostate gland depends on reciprocal cellular interaction between prostate epithelium and its adjacent stroma (Chung et al., 1991; Cunha et al., 1996; Wong and Wang, 2000;

Wong et al., 1998). Evidence also suggests that androgen receptor in the stroma rather than in the epithelium may be critical for conferring the growth and differentiation functions of the prostate gland (Cunha and Chung, 1981; Thompson and Chung, 1984). When normal prostate epithelium (Chung, 1995; Zhau et al., 1994) or urothelium (Zhau et al., 1994) was used in these studies, the inductive fetal urogenital mesenchyme determined the ultimate size of the tissue-tissue recombinant. However, when prostate tumor tissues (Chung et al., 1984; Miller et al., 1985) or tumor cells derived from the prostate (Gleave et al., 1991; Gleave et al., 1992) or urinary bladder (Zhau et al., 1994) were used in the experiments, the growth of the tissue-tissue or tissue-cell recombinants was uncontrolled and never reached a state of homeostasis.

One interpretation of these results is that signaling between tumor and stroma is aberrant and resembles a "vicious cycle" where a dysfunction of cytokine trafficking exists between tumor and host cells (Mundy et al., 2001; Tester et al., 2002). There are several possible mechanisms for the activation of a vicious cycle between tumor and stroma: 1) tumor cells secrete putative cytokines, growth factors and/or extracellular matrices that alter the morphology and gene expression of surrounding stroma such that the altered stroma becomes highly inductive and reciprocally induces the growth and gene expression of tumor epithelium thus initiating the vicious cycle. Rowley and collaborators provided evidence that stromal fibroblasts surrounding tumor epithelium underwent trans-differentiation to become a morphologically and biochemically distinct population of myofibroblasts (Rowley, 1998; Tuxhorn et al., 2001). Interestingly, they have shown that this type of stromal response to tumor epithelium can predict PSA-free survival in patients with prostate cancer (Burchardt et al., 2000; Joseph et al., 1997). 2) Tumor cells secrete soluble factors that act in an autocrine manner to promote the vicious cycle regardless of the surrounding stroma. Under certain stress and androgen conditions, increased growth factors, such

as vascular endothelial growth factor (VEGF) production by tumor cells, have been observed (Jackson et al., 2002; Wong et al., 1998). Increased VEGF was shown to induce more oxygen stress and initiate the vicious cycle by promoting more VEGF production by tumor cells, eventually causing an accumulation of neovasculature surrounding the tumor epithelium (Arbiser et al., 2002; Burchardt et al., 2000; Colavitti et al., 2002; Ferrer et al., 1997; Ferrer et al., 1998). 3) The intrinsic genetic instability of tumor cells can be promoted by tumor-microenvironment interaction (Rhee et al., 2001; Tlsty, 2001). It is conceivable that increased cytogenetic changes, with loss or gain of growth control genes, could fuel additional genetic instability not only of tumor cells per se but also of their surrounding stroma (Moinfar et al., 2000).

Potential factors responsible for activating prostate carcinogenesis and driving the "vicious cycle" of prostate stroma and tumor

Integrins. Integrins are important in prostate cancer progression and metastasis. The major role of integrins in cancer is the "outside-in" pathway, in which integrin activation induces cancer cell migration and invasion. Integrins also cooperate with growth factors to promote cell proliferation. When adherent tissue cells are released from their surrounding extracellular matrix, they forfeit survival signals and undergo apoptosis (Porter and Hogg, 1998). In addition to interacting with stromal cells or ECM, integrins can also form cis associations with other receptors on the same cell, forming multi-receptor complexes. These complexes recruit signaling molecules to sites of cell-cell or cell-matrix adhesion, such as focal complexes and focal adhesions (Edlund et al., 2001). Integrins also play a crucial role in regulating the actin cytoskeleton at the site of contact with ECMs. Although the detailed pathway is still not very clear, data show that once integrins receive signals from ECMs they can turn on other genes such as α-actin, talin, vinculin

and vasodilator-stimulated phosphoprotein. Signaling through Rho family pathways could activate Cdc42, Rac and Rho genes which further turn on downstream signaling pathways such as the calpain and JNK pathways. Integrins also could regulate the activation of the focal adhesion kinase pathway, Src protein tyrosine kinase, and paxillin, which are important in the remodeling and turnover of adhesion complexes (Martin et al., 2002).

Modulation of integrin activation is closely linked to gene expression, cell cycle progression and cellular behaviors, such as cell motility, migration and survival under various physiologic and pathologic conditions. The increased expression of $\alpha 3$ and $\alpha 6$ integrins compared with normal cells has been demonstrated. The expression of $\alpha 6\beta 1$ integrin on prostate cancer cells was linked to increased invasion of prostate cancer in a mouse model (Schmelz et al., 2002). We and other groups have studied cell interactions with extracellular matrix and stromal factors during disease progression by characterizing integrin usage and expression in a series of parental and lineage-derived LNCaP human prostate cancer cell lines (Allen et al., 1998; Bello-DeOcampo et al., 2001; Cooper et al., 2002; Cress et al., 1995; Edlund et al., 2001; Schmelz et al., 2002). Although studies indicated the decrease of integrin heterodimers, the actual integrin expression on the cell surface showed no significant change; however, with disease progression, integrin usage did change significantly. The more metastatic sublines were distinct in their use of $\alpha v\beta 3$ integrin (Edlund et al., 2001). When compared with parental LNCaP cells, the more metastatic sublines showed a shift in $\alpha 6$ heterodimerization, a subunit critical not only for interaction with prostate basal lamina but also for interaction with bone matrix proteins, a favored site of prostate cancer metastases (Edlund et al., 2001). This indicates that integrin usage changed during the progression of prostate cancer. The activation state of integrins could be an important element in how cells adapt under different microenvironmental conditions. The adaptive property of integrins could be

enhanced further with changes in mediators or ECMs during the metastatic progression of prostate tumor epithelial cells, a potential step toward migratory properties.

The αvβ3 integrin heterodimer has been detected on many different cell types, such as macrophage, endothelial cells, osteoclasts, and cancer epithelial cells. The activation of αvβ3 in prostate cancer cells is mediated by the FAK pathway that activates the downstream PI-3 kinase/Akt pathway (Zheng et al., 2000). This triggers alterations in cell adhesion and migration on a variety of extracellular matrix proteins, including vitronectin, fibronectin, fibrinogen, laminin, collagen, and osteopontin. αvβ3 has been shown to be important for prostate cancer bony metastasis by adhesion of cancer cells to bone matrix components, vitronectin, osteopontin and bone sialoprotein (BSP). Through analysis of DU145 cell adhesion to ECM, Zheng and colleagues showed that the adhesive property of DU145 cells can be decreased by LM609, a blocking αvβ3 antibody (Zheng et al., 1999; Zheng et al., 2000). Osteopontin and vitronectin are common proteins in mature bone and can potentially serve as ligands for αvβ3.

Integrin-associated protein (IAP/CD47) is a 50 kDa single-chain protein composed of an extracellular immunoglobulin superfamily (IgSF) domain, five membrane-spanning sequences and a short cytoplasmic tail. IAP was first isolated as a protein associated with the integrins, αvβ3, αIIbβ3, αvβ5 and α2β1. Human cells that lack IAP are deficient in αvβ3mediated ligand binding (Porter and Hogg, 1998). In an affinity purification study in which the 179-208 peptide of the α3(IV) chain of collagen IV was used as the immobilized element, five proteins from melanoma and prostate cells were isolated. The 3 proteins were shown to be CD47/IAP, the integrin β3 subunit, and the αvβ3 integrin complex, respectively (Shahan et al., 1999), indicating that αvβ3 and IAP formed a complex in prostate cancer cells that could activate the functional property of

αvβ3 in prostate cancer epithelial cells. Another recent study indicated that αvβ3 inhibits endothelial cell apoptosis during angiogenesis through NF-κB activation (Cooper et al., 2002). Angiogenesis facilitates the growth and metastasis of tumors by providing support and facilitating cancer migration. Together these studies suggest that αvβ3 is important not only for the growth and survival of tumor epithelium but for its supporting endothelium.

Growth factors. The expression of *basic fibroblast growth factor (bFGF, FGF-2)* has been shown to be significantly increased in stromal fibroblasts in human prostate cancer and in endothelial cells compared with normal tissue. Prostate carcinoma cells have been shown to up-regulate fibroblast growth factor receptor isoforms with a high affinity for bFGF during cancer progression (Dow and deVere White, 2000). Accordingly, elevated sensitivity bFGF may stimulate cancer cell proliferation and protease expression, thereby supporting tumor growth and invasion. In addition, overexpression of both FGFR-1 and FGFR-2 in prostate cancer epithelial cells in a subset of prostate cancers has been correlated with poor differentiation. Thus, there is both an increase in bFGF concentration in stromal cells and increased expression of receptors in tumor epithelial cells which respond to bFGF, establishing a potential paracrine loop between prostate cancer cells and their surrounding stromal cells, which may be important for prostate cancer progression (Giri et al., 1999).

bFGF also stimulates fibroblast proliferation and extracellular matrix turnover through increased deposition and protease degradation (De Benedetti and Harris, 1999; Dow and deVere White, 2000), and functions as an angiogenic factor that induces endothelial cell migration, proliferation and differentiation into new blood vessels (De Benedetti and Harris, 1999). Thus bFGF may promote prostate cancer progression by inducing angiogenesis and stromal remodeling. A study also indicates the increase of bFGF in tumor epithelial cells due to induction of stromal

FGF-2 (Giri et al., 1999), thus potentially establishing a positive feedback loop. Human prostate cancer cell lines DU-145 and PC-3 have been shown to express FGF-2 and metastasize to bone (Dow and deVere White, 2000). Furthermore, studies of the Dunning rat model show that activation of bFGF expression accompanied progression of epithelial cells to malignancy (Yan et al., 1993). These data suggest a possible contributing role for bFGF in the vicious cycle of tumor formation and progression.

Platelet-derived growth factor (PDGF) is a 30-kD protein consisting of disulfide-bonded homodimers or heterodimers of A and B subunits, also designated as c-sis (Sitaras et al., 1988). Its isoforms have been indicated as important during embryonic development, particularly in the formation of connective tissue in various organs (George, 2001). In adult tissues, the primary function of PDGF is to stimulate wound healing via chemotaxis and mitogenesis of fibroblasts, and secretion of extracellular matrix components (Tuxhorn et al., 2001). The normal physiologic targets for PDGF are stromal cells such as fibroblasts, endothelial cells, smooth muscle cells, and glial cells. Thus, paracrine release of PDGF stimulates stromal reactions in normal and pathologic states. Receptor binding by PDGF is known to activate intracellular tyrosine kinase, leading to autophosphorylation of the cytoplasmic domain of the receptor as well as phosphorylation of other intracellular substrates. This reaction is described as one in trans, i.e., the two receptor molecules of the receptor dimer phosphorylate each other. Specific substrates identified with the beta-receptor include Src, GTPase Activating Protein (GAP), phospholypase C (PLC) and phosphotidylinositol 3-phosphate. Both PLC-γ and GAP seem to bind with different affinities to the α- and β-receptors, suggesting that the particular response of a cell depends on the type of receptor it expresses and the type of PDGF dimer to which it is exposed. In addition to the above, a non-tyrosine phosphorylation-associated signal transduction pathway can also be activated that

involves the zinc finger protein Erg-1 (Khachigian and Collins, 1998).

Immunohistochemical analysis of PDGF and PDGFR indicated expression in both prostate epithelial and stromal cell types and in PIN lesions (Fudge et al., 1996). In contrast, the normal epithelial cells do not express PDGF nor PDGFR (Fudge et al., 1994). In vitro study of PDGF indicates that release of PDGF from tumor cell lines stimulates prostate stromal cell proliferation (Vlahos et al., 1993). This suggests that de novo expression of PDGF occurs early in prostate tumor progression. The production and activation of PDGF could further enhance the stromal reaction and contribute to the vicious cycle of tumor progression.

Recruitment of new blood vessel growth clearly illustrates the importance of carcinoma-stromal interactions during cancer progression. In normal human prostate tissue, *VEGF* is reportedly expressed at low levels and restricted to stromal cells. In high-grade PIN and prostate cancer, elevated expression of VEGF was observed in cancer, stroma and vascular endothelium (Ferrer et al., 1997). Endothelial cells from microvessels in the surrounding stroma must be induced to migrate into the tumor, whereby they proliferate and form new blood vessels to support tumor growth. This complex process is regulated by a delicate balance of angiogenesis inducers and angiogenesis inhibitors in the extracellular milieu. Increased activator(s) and/or decreased inhibitor(s) alter the balance and lead to the growth of new blood vessels (Hanahan, 1997). Several growth factors, such as VEGF, PDGF, TGF-β and connective tissue growth factor, from epithelium or stroma, could induce angiogenesis (Nadal et al., 2002).

Recent studies indicate that VEGF directly stimulates prostate tumor cells via autocrine and/or paracrine mechanisms (Jackson et al., 2002; Sokoloff and Chung, 1998). One example demonstrated the possible role of VEGF as a mediator in the vicious cycle of tumor and stroma, in which reactive oxygen species (ROS) could participate in early prostate cancer epithelium growth

and development. Increased ROS could enhance the production of VEGF, further promoting ROS concentration in stromal fibroblasts. The resulting overexpression of VEGF from stromal fibroblasts could induce Nox1, MMP-9, VEGF, and VEGFR production and increase the overall tumor growth rate (Arbiser et al., 2002).

In normal prostate tissue, *IGF-1* is produced only by stromal cells, while prostate epithelial cells express insulin-like growth factor binding proteins (IGFBP-2, 3, 4, and 6) and the type 1 IGF receptor (Lopaczynski et al., 2001). It has been shown that both bFGF and PDGF can enhance IGF-1 production from endothelial cells. Prostate cancer patients have shown increased serum IGF-1 and a decrease of IGFBP-3 level (Chan et al., 1998; Chan et al., 2002; Chokkalingam et al., 2002; Grimberg et al., 2002; Mantzoros et al., 1997). The increased serum IGF-1 concentration in prostate cancer patients could be from the stromal cells, metastatic prostate cancer epithelial cells or the liver. However, the study of IGF secretion is controversial. Some reports did not detect IGF-1 production by prostate cancer cells (Angelloz-Nicoud and Binoux, 1995; Cohen et al., 1991; Connolly and Rose, 1994; Pietrzkowski et al., 1993). Others indicated the expression of IGF by prostate cancer cells (Iwamura et al., 1993; Kaplan et al., 1999; Kimura et al., 1996). Interactions between the glandular epithelium and the myofibroblasts and fibroblasts of the stromal compartment of the prostate gland appear to be regulated by IGF-1 *availability*. IGF-1 may act directly through the androgen receptor pathway, and may be regulated through EGF-TGF-α receptor regulatory signaling (Kimura et al., 1996). This suggests the possible vicious cycle of IGF-1 production in prostate cancer progression. First, release of bFGF and PDGF induces IGF-1 secretion from prostate stromal cells, which could induce the increased production of androgen receptor and/or EGF from prostate tumor epithelial cells. The enhanced expression of the androgen receptor and elevated release of EGF can in turn further stimulate the

release of IGF-1 from stromal cells, which may promote the progression of prostatic carcinoma cells.

Two huge molecules called *plasminogen-related growth factors (PRGFs)*, evolutionarily related to plasminogen, play an important role in inducing invasive growth of cancer progression. PRGF-1 is also called *hepatocyte growth factor/scatter factor (HGF/SF)*. PRGF-2 is also known as *macrophage-stimulating protein (MSP)*, scatter factor-2 (Comoglio et al., 1999). HGF/SF has been demonstrated to be important in prostate cancer progression and metastasis, while MSP may be an important neurotrophic factor in embryonic development by inducing superoxide anion production (Brunelleschi et al., 2001; Rampino et al., 2002). It has been shown that both HGF/SF and MSP were up-regulated in the wound repair process in a rat model (Cowin et al., 2001). HGF/SF predominantly participates in a paracrine network. Several mesenchymal-derived cells (fibroblasts) secrete HGF/SF. It has been implicated as a mediator involved in communication between epithelial cells and the microenvironment (Comoglio and Trusolino, 2002). HGF/SF is secreted predominantly by stromal fibroblasts and stimulates proliferation and migration of epithelial and endothelial cells during organ development and tissue remodeling (Parr and Jiang, 2001). The secretion of HGF/SF as an inactive pro-HGF, which is converted into its bioactive form by a proteolytic cleavage by four proteases: urokinase (uPA), serine protease in the serum, coagulation factor XII, and its homologues (Comoglio et al., 1999). It has also been shown that some epithelial cells secrete two potent inhibitors of pro-HGF activation (HAI-1 and -2) that tightly control HGF/SF activation (Denda et al., 2002).

In normal prostate epithelial cells, HGF/SF secreted by stromal cells causes growth inhibition, sustained phosphorylation of mitogen-activated protein kinase, and increased gene expression consistent with cell differentiation. Several soluble factors increase the production of

HGF/SF in myofibroblasts but not in normal prostate epithelial cells, such as IL-1β, PDGF, bFGF, VEGF, and EGF (Zhu and Humphrey, 2000). Increased expression of the HGF/SF receptor c-Met proto-oncogene has been associated with progression of several types of carcinoma, including that of the prostate (Humphrey et al., 1995; Pisters et al., 1995; van Leenders et al., 2002). As mentioned above, the vicious cycle of bFGF, PDGF, and VEGF could further mediate or enhance the secretion of HGF/SF from stromal cells. Several studies have indicated that the increased concentration of HGF/SF in ECM could further contribute to malignancy in DU145 or PC-3 prostate tumor cells, inducing migration (Gmyrek et al., 2001; Nishimura et al., 1999).

Cytokines

In a normal homeostatic state, *IL-6* levels are typically very low. However, in response to microenvironment inflammatory factors, IL-6 can be released by wide variety of cell types. Cells known to express IL-6 include CD8+ T cells, fibroblasts, synoviocytes, adipocytes, osteoblasts, megakaryocytes, endothelial cells (under the influence of endothelins), neurons, neutrophils, monocytes, colonic epithelial cells, and B cells. IL-6 production is generally correlated with cell activation. Studies of tumor stroma indicate that increased IL-6 could induce the progression of prostate tumor epithelial cells by inducing the release of other cytokines, bone resorption, and induction of thrombopoiesis (Smith et al., 2001). Several groups have reported elevated serum levels of IL-6 upon progression of prostate cancer to androgen-independence (Nakashima et al., 2000; Shariat et al., 2001). It is possible that mediators released from prostate tumor epithelial cells could further enhance the production of IL-6 from both stromal and inflammatory cells. The increase concentration of IL-6 in ECM may further induce prostate tumor epithelial cells to produce mediators and IL-6R. Once prostate cancers reach malignancy, the tumor epithelial cells

can produce IL-6 themselves and form an active autocrine loop.

A recent study indicated the increase of both IL-6 and IL-6R in prostate tumor epithelial cells with the increase of malignancy (Giri et al., 2001), again suggesting a "vicious cycle" mediated by IL-6 during the early development of prostate cancer and becoming an increasingly active autocrine loop in highly metastatic tumors. Similar vicious cycles have been shown for other cytokines, such *IL-8*. In human prostate cancer, IL-8 has been shown to stimulate PC-3 prostate cancer cell migration and invasion in vitro through a reconstituted basement membrane and both long-term migration and short-term adhesion to laminin (Reiland et al., 1999). IL-8 is produced by many different stromal cells, including endothelial cells. IL-8 is also produced by various metastatic tumor cells, including prostate cancer cells (Kim et al., 2001). Furthermore, stress factors, such as hypoxia, acidosis, nitric oxide (NO), and cell density, which increase with the progression to malignancy, can also influence IL-8 production (Shi et al., 2000).

Stromal response to early prostate inflammatory atrophy

Inflammatory reactions often result in the activation and recruitment of phagocytic cells (e.g., neutrophils and/or tissue macrophages) whose products, such as cytokines, oxidants and free radicals, result in injury to the tissue. Recent reports indicate that benign prostatic hyperplasia (BPH) frequently exhibits infiltration of CD4(+)/CD45RO(+) memory T-lymphocytes. This infiltration could induce growth of myofibroblast cells in BPH. Increased level of cytokines, such as IL-2, IL-4 and INF, were also detected in T-cells in BPH but not in normal prostate (Kramer et al., 2002). Another study also indicated the association of inflammation with BPH and prostate cancer, and the increased expression of Bcl-2 in these prostate patients (Gerstenbluth et al., 2002). The chronic inflammation status linked to the development of carcinoma has been reported in

several organ systems, including prostate cancer (De Marzo et al., 1999). The hypothetical mechanism involves repeated tissue damage and regeneration in the presence of highly reactive oxygen. These reactive molecules, i.e. hydrogen peroxide (H_2O_2) released from the inflammatory cells interact with DNA in the proliferating epithelium to produce permanent genomic alteration, such as frame-shift mutation, deletions, and rearrangements, as well as increasing the epithelial proliferative rate (Gasche et al., 2001; Oda et al., 2001). Recent studies of prostate cancer indicated inflammatory responsive cells at the juxtaposition of highly proliferative prostate epithelial cells, referred to as a Proliferative Inflammatory Atrophy lesion (PIA lesion). Studies demonstrated mononuclear and/or polymorphonuclear inflammatory cells in both the epithelial and stromal compartments, and stromal atrophy with variable amounts of fibrosis. Luminal epithelial cells of PIA lesions have elevated levels of Bcl-2, decreased expression of $p27^{Kip1}$, and increased levels of π-class glutathione S-transferase (GSTP1) (De Marzo et al., 1999). In vivo study of H_2O_2 and GSTs indicated that H_2O_2 enhances the expression of GSTP1 (Liu et al., 2001). This indicates that increase expression of GSTs in PIA may be due to increased concentrations of H_2O_2 in the stromal microenvironment during the PIA stage. However, PIN and prostate cancer cells rarely express GSTα isoenzyme (GSTA1) and GSTP1 as a result of increased methylation of GSTP1 in a "CpG island" which inactivates GSTP1 (De Marzo et al., 1999; Parsons et al., 2001).

The phenotypic switch of stromal cells, extracellular matrix remodeling, increased growth factor availability, elevated protease activity, increased angiogenesis and recruitment of inflammatory cells were observed in cancer progression. The stromal response to cancer shows similarity to the wound repair response (Tuxhorn et al., 2001), and it is possible that these conditions could promote tumorigenesis. The phenotypic switch between fibroblast and myofibroblast indicates increased extracellular matrix remodeling during prostate cancer

progression. In normal prostate it has been reported that the "stromal network of collagen fibers is loosely woven, fine and smooth in texture," while in Gleason-score seven adenocarcinoma the collagen fibers "appeared swollen in diameter" and there was "no regularity in the spatial relationship of the fibers" (Keller et al., 2001; Tuxhorn et al., 2001). This suggests that remodeling of the extracellular matrix is one of the key features of stromal reaction in prostate cancer.

Prostate Cancer and Bone Stromal Interaction

The progression of prostate cancer from the androgen-dependent to the androgen-independent and bone metastatic state is considered a poor and generally lethal prognosis. To understand the molecular basis of disease progression and develop rational new therapeutic approaches for targeting prostate cancer bone metastasis, we must first understand the multi-step processes that lead to prostate cancer metastasis to bone. As depicted in Figure 2, at the site of primary cell growth we expect prostate cancer cells to interact with prostate stromal cells and gain the ability to extravasate into the bloodstream. In the blood, prostate cancer cells are expected to survive and move as an embolus prior to adhering to bone marrow-associated endothelial cells. The attachment and interaction of prostate cancer cells to marrow endothelial ECMs could activate the invasive properties of prostate cancer cells and allow their extravasation into the marrow space. At the final step of this progression, prostate cancer cells interact directly with osteoblasts and osteoclasts through a series of soluble factors (e.g., receptor activator of NF-κB ligand, RNAKL) via cell surface receptor (e.g., RNAK) to survive, proliferate, migrate and invade and eventually replace the bone marrow components. To understand the cellular and molecular basis of the prostate carcinoma-bone stroma interaction, it is essential to delineate how the soluble growth factors and extracellular matrices participate reciprocally in the progression of prostate cancer

toward androgen-independence and bone metastasis.

A conceptual framework will be introduced here to illustrate the following issues. 1) Bone stromal reaction to cancer epithelium may signal further tumor progression. Altered bone stromal cells, in response to tumor epithelium, may induce further epithelial genetic and phenotypic changes and thus contribute to a vicious-cycle cascade in the androgen-independent and metastatic progression of prostate cancer (see Figure 1). 2) Co-targeting tumor and stroma could starve or kill tumor cells from their supporting microenvironment, and could offer the greatest benefits for inducing tumor regression and sustaining the long-term survival of patients with prostate cancer skeletal metastasis and its associated complications.

By using a human prostate cancer co-culture model, our laboratory has obtained evidence suggesting that non-random genetic changes occur in human bone stromal cell line MG-63, after co-culturing with the human androgen-independent prostate cancer cell line C4-2 (a lineage-derived LNCaP subline with growth and metastatic potential to lymph node and bone when injected subcutaneously or orthotopically in castrated mice) under 3-D conditions. The 3-D model is valuable for the evaluation of the prostatic carcinoma-bone stroma interaction in vitro. The participation of bone stroma in tumor growth and progression suggests that when prostate cancer metastasizes to bone, there are complex and reciprocal cellular interactions between populations of tumor and host bone cells.

"Vicious Cycle" between Prostate Cancer and Bone Stroma

Laboratory observations

While clinical human prostate cancer is predominantly osteoblastic, the established human prostate human cancer cell lines inoculated and grown in the bone of immune-compromised mice

yield both osteoblastic and osteolytic lesions. Apparently, prostate cancer cells can participate in the process of bone turnover by exhibiting properties similar to osteoblasts, the so-called "osteomimetic" properties of prostate cancer cells as reported earlier (Koeneman et al., 1999). Much evidence supports this interesting phenotype of prostate cancer cells, in which they behave like osteoblasts. Prostate cancer cells express both soluble and membrane-bound RANK ligands, and were shown to participate directly in osteoclastogenesis (Koeneman et al., 1999; Matsubara et al., 2001; Yeung et al., 2002; Zhang et al., 2001). Prostate cancer cells expressed a number of non-collagenous bone matrix proteins, such as osteocalcin, osteopontin, osteonectin and bone sialoprotein, alkaline phosphatase, and a key transcription factor, Runx 2 (cbfa1) that controls the transcription of osteocalcin and collagenous-3 (D'Alonzo et al., 2002). In addition, upon exposure to mineralizing cell culture conditions, prostate cancer cells have been shown to form bona fide mineralized bone crystals as detected by electron microscopy (Lin et al., 2001). These observations raise the possibility that soluble and/or matrix-associated molecules may be responsible for signaling between prostate cancer and bone stromal cells. Since bone-homing prostate cancer cells seek to adhere, colonize and survive in bone, it is of pivotal importance to find out how prostate tumor and bone cells interact with the hope of identifying novel therapeutic targets for the treatment of prostate cancer bone metastasis. One attractive hypothesis is that prostate cancer cells may behave like osteoblasts and functionally participate in bone turnover. By markedly increasing the basal rate of bone turnover, this may further enhance prostate cancer cell colonization in bone (Cher, 2001; Nemeth et al., 2002). This hypothesis is supported by some clinical observations, where bisphosphonates, an effective class of agents that slow down or inhibit bone resorption, have been shown to reduce cancer cell colonization in experimental models of prostate and breast cancers (Coleman, 2001; Lee et al., 2001). In men harboring prostate cancer,

there is evidence that increased bone resorption occurs upon castration. Whether these changes in bone turnover subsequent to hormonal manipulation or bisphosphonate treatment after prostate cancer cell colonization in bone affect the natural history of prostate cancer progression should be the subject of future thorough investigation.

Factors driving the "vicious cycle" between prostate cancer and bone cells

Guise and colleagues (Chirgwin and Guise, 2000) and Mundy (Mundy, 2002) presented the concept of a "vicious cycle" involving TGF-β produced by bone cells that promotes the production of PTHrP by tumor cells, which in turn stimulates bone turnover by enhancing osteolytic reaction in the bone. TGF-β is released as a result of rapid bone turnover, which in turn will trigger increased PTHrP production by cancer cells. The production of PTHrP by tumor cells will induce osteolytic cells to express an increased level of RANK ligands, which can promote osteoclast formation/activation and subsequently increased bone resorption. The enhanced resorptive process by osteoblasts and osteoclasts leads to "bone pitting" and subsequent colonization by cancer cells in the skeleton, and associated bone destruction often observed in cancer patients. Thus, a "vicious cycle" may exist between TGF-β, PTHrP, RANK ligands in osteolytic prostate cancer. Interrupting the vicious cycle in cancer models using anti-PTHrP antibodies or osteoprogerin (OPG) has been shown to reduce colonization of cancer metastasis to bone (Zhang et al., 2001).

Transforming growth factor-β (TGF-β) is a 25-kDa disulfide-linked polypeptide which coordinates cell function over distances by binding to cell surface receptors. An immunohistochemical study of mouse prostate development indicated that TGF-β1 is expressed in mesenchymal cells (Timme et al., 1995). It was initially characterized by its effects on epithelial

function and proliferation (Cui et al., 1995), but it is also an important mediator of stromal reaction (Wakefield and Roberts, 2002). Responses to TGF-β include phenotypic changes affecting adhesion, migration, differentiation and cell fate. In general, TGF-β stimulates the production of ECM components, inhibits degradation, and alters integrin expression. It follows that all of these effects can significantly alter cell behavior.

TGF-β is abundant in latent forms that circulate or are bound to the ECM in bone. Activated TGF-β can bind to ubiquitous heterodimeric receptors and induce signal cascade through the SMAD pathway (Taipale et al., 1998). Stromal and epithelial cells of malignant and nonmalignant prostatic tumors express all three TGF-β isoforms and their related receptors which act as paracrine and autocrine factors, influencing prostate function and stromal-epithelial cell interaction (Cardillo et al., 2000). These data indicate that TGF-β1 produced by carcinoma cells acts on the surrounding stromal cells, which in turn induces stromal cells to release cytokines to further promote the malignancy of the cancer cells.

In addition to the TGF-β and PTHrP connection, a number of other candidate molecules may also contribute to the vicious cycle of cancer growth and bone metastasis. For example, bone is a rich source of hydrogen peroxide, and hydrogen peroxide has been shown to increase the production of vascular endothelial growth factor (VEGF) by tumor cells. There is evidence that increased VEGF could further stimulate increased production of hydrogen peroxide by tumor and bone cells. Since VEGF is known to be required to support tumor growth and colonization, it is possible that a hydrogen peroxide/VEGF connection contributes to the vicious cycle between tumor and bone cells (Arbiser et al., 2002).

Endothelium-1 (ET-1) may also contribute to osteoblastic reaction when prostate cancer cells colonize to bone. ET-1 production is negatively regulated by androgen. Thus castration

could potentially reduce osteoblastic reactions in bone through the reduction of ET-1. However, ET-1 and its interaction with receptor ET-1A could participate in the osteoblastic reaction and spur the vicious cycle in prostate cancer and bone by increased production of cytokines such as IL-1α, IL-1β, EGF, TNF-α, and TGF-β (Granchi et al., 2001; Le Brun et al., 1999). Increased production of TGF-β, EGF and IL-1α has been shown to upregulate ET-1 in PC-3 and DU145 cells, hence altering the growth factor and cytokine milieu in bone in response to ET-1 growth factor (Granchi et al., 2001). Cytokines may contribute to further prostate cancer growth and colonization to bone.

ET-1, composed of 21 amino acid residues, was originally isolated from porcine aortic endothelial cells (Kurihara et al., 1989). ET-1 is one of the four families of vasoactive peptides that include endothelin-2 (ET-2), endothelin-3 (ET-3), and endothelin-4 (ET-4) (Cunningham et al., 1997). All members of the endothelin family contain two essential disulfide bridges and six conserved amino acid residues at the C-terminus. In addition, they all are synthesized as pre-pro-polypeptides which need to be cleaved to produce pro-polypeptides. The pro-ET-1 is proteolytically cleaved by a membrane-bound metalloproteinase, endothelin-converting enzyme (ECE-1), produced by endothelial and epithelial cells (Xu et al., 1994). Two receptors for endothelins have been characterized, designated ETA and ETB. Although these receptors are structurally and functionally different, they share some similarities. Both are seven membrane domain receptors coupled through G proteins to phospholipase C. Both have an N-terminal signal sequence and a long N-terminal extracellular domain (Sakurai et al., 1992). ETA shows a higher affinity for ET-1 than for ET-2 and the lowest affinity for ET-3. The ETB receptor shows approximately equal affinity for each of the endothelins. Both ETA and ETB have been identified in prostate tissue. Stroma has higher concentration of ETA, while ETB is predominately in the epithelial cells of the prostate (Remuzzi and Benigni, 1993).

In human prostate cancer progression, ET-1 and ETA expression is retained, whereas ETB receptor expression is reduced. ET-1 protein expression was detected in situ in 14 of 14 primary cancers and 14 of 16 metastatic sites. Exogenous ET-1 induces prostate cancer proliferation directly and enhances the mitogenic effects of IGF I, IGF II, PDGF, bFGF, and EGF in serum-free conditions in vitro. ETA antagonist A-127722 inhibits ET-1-stimulated growth, but the ETB-selective receptor antagonist BQ-788 does not. ET-3, an ETB-selective agonist, also had no effect on prostate cancer growth. No specific ETB-binding sites could be demonstrated in any established human prostate cancer cell line tested, and ETB mRNA, detected by reverse transcription PCR, was reduced. The predominance of ETB binding in human benign prostatic epithelial tissue is not found in metastatic prostate cancer by autoradiography. Furthermore, a study of ET-1 in prostate cancer bone metastasis demonstrated that ET-1 is mitogenic for osteoblasts, inhibits osteoclastic bone-resorption and induces the formation of osteoblastic lesions. All this suggests that ET-1 is involved in the new bone formation associated with prostate cancer metastasis (Nelson et al., 1999).

Tests of the mitogenic property of ET-1 indicated that other factors also can be co-factors with ET-1, such as bFGF and IGFs. PDGF and ET-1 also can play a role in tumor angiogenesis in conjunction with VEGF. Clinical trials of ET-A receptor antagonist in prostate cancer indicated that it could help patients, if they could tolerate mild but pervasive symptoms related to ET-1's vasoconstrictive effects (Kopetz et al., 2002).

A recent study (Taichman et al., 2002) showed that stromal chemokine and receptor, such as stromal cell-derived factor-1 (SDF-1 or CXCL12) and its receptor (CXCR4), may play a role as prostate cancer bone metastasis homing signals. The level of CXCR4 increased with the malignancy of the prostate cancer cell lines by both RT-PCR and Western blot analysis. The

increased expression of CXCR4 also increased spreading to bone in animal studies. An in vitro study of cellular spreading in basement membrane indicates that spreading can be inhibited by CXCR4 antibody. These findings suggest that chemokine and its receptor could also be important in prostate cancer bone metastasis.

Together these studies indicate that the process of prostate cancer bone metastasis is a complicated pathway requiring multiple chemokines, cytokines and membrane proteins. These complexities also suggest the possibility of therapeutic strategies specifically focused on co-targeting and disrupting key carcinoma-stroma interactions.

Cancer Therapy Based Upon Co-Targeting Tumor and Stroma

Laboratory and clinical observations

Because prostate cancer growth is highly susceptible to tumor-microenvironment interaction and experimentally can be promoted by stromal fibroblasts, it is reasonable that control of prostate tumor growth might be optimized by co-targeting both tumor and stroma. To explore this concept, we designed studies to co-culture prostate cancer cells and bone stroma in vitro, establishing chimeric tumor models consisting of human prostate cancer cells and bone stroma. By introducing a "bystander" therapeutic gene, herpes simplex thymidine kinase (hsv-TK), to stromal cells only, we observed effective cell kill in tumor epithelium in vitro and shrinkage of tumor size in vivo upon addition of a pro-drug, gancyclovir (GCV). Since there were no identifiable gap junctions between prostate tumor cells and bone stroma under the electron microscope, we concluded that there must be metabolic cooperation between tumor epithelium and bone stroma mediated by soluble factors and extracellular matrices. By interrupting this communication, and targeting both tumor and stroma, tumor growth and survival may be adversely affected. Conceptually,

co-targeting tumor and stroma in prostate cancer bone metastasis is a rational approach to the "vicious cycle" constantly operating between tumor and stroma. Directly inducing cell-kill of tumor epithelium and starving cancer cells by disrupting tumor interaction with the stromal compartment could achieve the best possible tumor regression.

In our laboratory, we co-targeted tumor and stroma using an adenoviral vector in which therapeutic gene expression was controlled by a tissue-specific and tumor restrictive promoter, such as osteocalcin, osteonectin, or bone sialoprotein. These have been shown to be highly effective in inducing long-term tumor regression, and even some cure in pre-established tumor in the skeleton with administration of the adenovirus through the intravenous route (Hsieh and Chung, 2001; Hsieh et al., 2002; Matsubara et al., 2001). This concept of bone targeting to improve therapeutic effects has received clinical support. Tu and colleagues (Tu et al., 2001) reported a significant prolongation of patient survival by targeting bone with strontium 89 and prostate tumors with chemotherapy.

Molecular basis of co-targeting

The bone microenvironment was depicted by Paget over a century ago as a specialized "soil" that favors the metastasis of certain selective cancer cell types ("seed"). While the precise mechanism by which cancer cells home to bone is still unknown, several attractive ideas and hypotheses have been proposed. Bone must express certain chemo-attractants that selectively retain circulating cancer cells, and cancer cells must express cognate ligands or receptors allowing them to attach to bone marrow-associated endothelial cells, marrow stromal cells or osteoblasts, and/or respond to bone-derived growth factors, cytokines/chemokines or extracellular matrices. To metastasize to bone, cancer cells must be able to survive "hostile" circulatory compartments, including the blood

and lymphatic channels. The mere detection of cancer cells in blood or marrow stromal compartments may not reflect the "vitality" of cancer cells. Solakoglu, et al (Solakoglu et al., 2002) recently demonstrated that the outgrowth of cytokeratin-positive tumor cells from bone marrow can be detected in 81% of prostate cancer patients. Increased cell viability in patients correlated with increased cancer-related deaths.

From our use of prostate cancer cell lines as a model to study carcinoma-stroma interaction, we suggest that a switch of transcriptional factors must occur during the pathogenesis of prostate cancer. This biochemical switch could occur early, even when epithelial cells are still in the primary stage, since even then the expression of bone-like proteins such as osteocalcin, osteopontin, osteonectin and bone sialoprotein was detected. Considering how osteocalcin promoter in prostate cancer cells is regulated, a vicious cycle could occur at the level of transcription factor activation, wherein the coordinated activation of transcription factors by known soluble factors and ECM-integrin signaling culminates in the ability of prostate cancer cells to proliferate and survive in bone. Numerous links have been established between the up-regulation of transcription factors such as Runx-2 and the potential alteration of cellular behavior that could lead to increased cell growth and spread to bone.

Runx-2 is a potent and specific transcription factor that controls mesenchymal-epithelial interaction in tooth development (D'Souza et al., 1999). Apparently, Runx-2 activation is controlled by soluble growth factors, and upon activation it can regulate soluble growth factor secretion, which ultimately controls the growth and differentiation of enamel tooth epithelium. Based upon this and other published data, we proposed that activation of similar transcription factors such as Runx-2 in prostate cancer cells could potentially enhance prostate cancer cell invasion and migration through the induction of collagenase (e.g., collagenase 3) and other

metalloproteinases. The concomitant induction of Runx-2, collagenase 3 and other growth and differentiation supportive factors could enhance prostate cancer survival and invasion. Similarly, the activation of the$\alpha V\beta 3$ and $\alpha 2V\beta 1$ integrin-ECM pathways may promote outside-in signals that result in enhanced cell migration and invasion.

We proposed earlier that prostate cancer metastasis to bone is not a random process. It involves the specific recognition of cancer cells by bone as "self" and the production of bone-like proteins by cancer cells. The expression of bone-like proteins by prostate cancer cells may allow them to adhere, proliferate and survive in the bone microenvironment and participate in certain normal functions of bone cells, i.e., bone resorption.

Cancer cells express a bone-like phenotype early, when they are in primary lesions. This raises the possibility that the expression of bone-like proteins by cancer cells and reactive stroma may serve as a prognostic biomarker for prostate cancer bone metastasis and possibly as a predictor for patient survival. By combining the expression of bone-like proteins and the stromal reaction to epithelium, it is possible that novel molecular markers can be developed both at the gene expression and genetic level. While the role of bone-like protein is presently unclear, it is possible that the activation of these processes may occur at the transcription level. Transcriptional factor switching could be of fundamental importance in determining the phenotype of cancer cells and might influence the extent of the vicious cycle between tumor cells and bone stroma. It is possible that specific targeting of transcriptional factors could have benefit as cancer therapy. Interrupting the activation of bone-like proteins in tumor epithelium and bone stroma may prevent prostate cancer cell adherence, proliferation and survival in bone.

Figure Legends

Figure 1: "Vicious cycle" of prostate cancer bone metastasis. This figure describes the growth factor and extracellular matrix-mediated activation of transcription factors, which control the matrix proteins, and MMPs activation. The vicious cycle may be initiated first by the presence of growth factor/ECM milieu in the prostate cancer which up-regulates key transcription factors that modulate matrix and metalloproteinase expression in stromal cells. Increased expression of chemokines, cytokines, and transcription factors can activate of additional growth factors and ECM pathways, which drive prostate tumor cells to a more invasive and malignant state.

Figure 2: The multi-step processes of prostate cancer metastasis to bone. Once prostate cancer cells gain the ability to extravasate into the bloodstream, prostate cancer cells move as embolus prior to adhering to bone marrow-associated endothelial cells. The attachment and interaction of prostate cancer cells to marrow endothelial ECMs could activate the invasive properties of prostate cancer cells and allow their extravasation into the marrow space. Prostate cancer cells than interact directly with osteoblast and osteoclast through a series of soluble factors via cell surface receptor (e.g., RNAK) to invade and replace the bone marrow components.

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Fig 1.

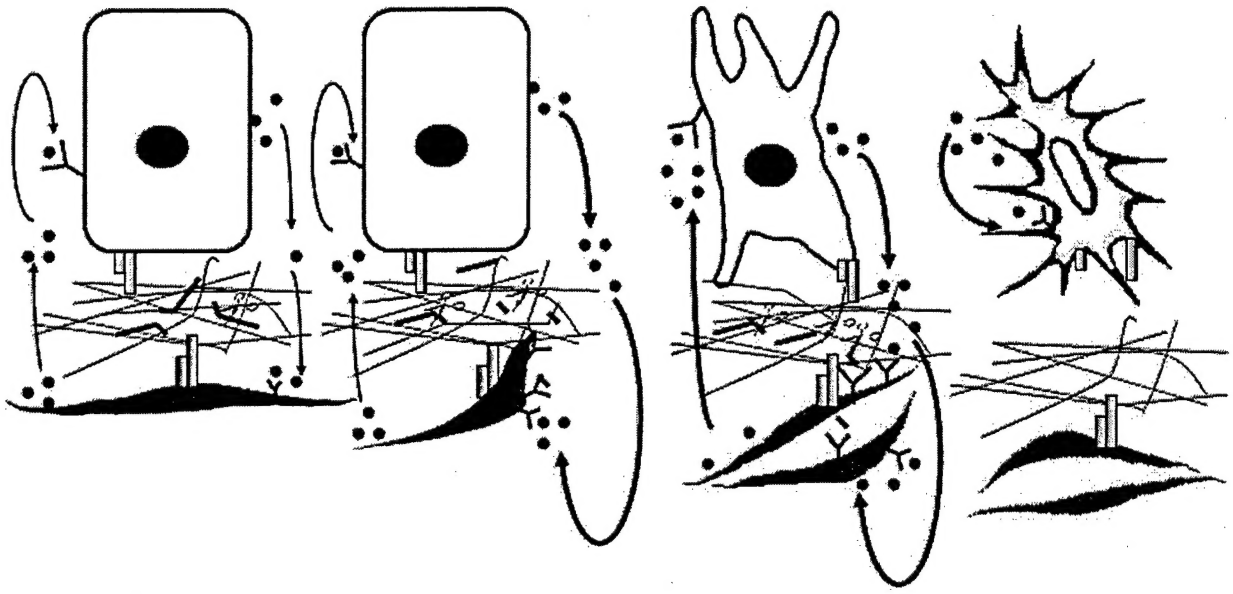
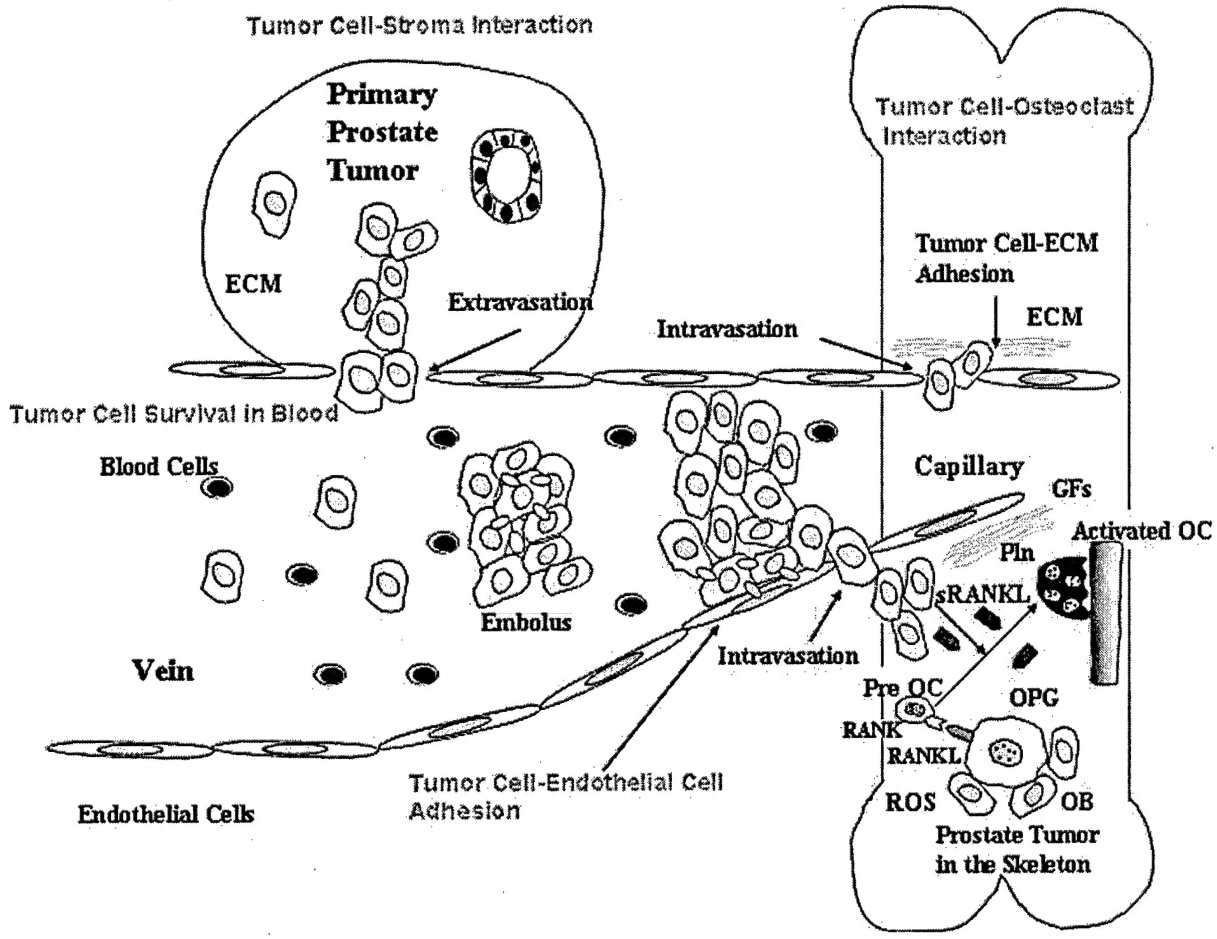

Stromal-Dependent
Reactive Stroma
Reactive Epithelium
Stromal-Independent
Androgen dependent

Fig 2.


Tumor Cell-Stroma Interaction
Primary Prostate Tumor
ECM
Extravasation
Intravasation
Tumor Cell-Osteoclast Interaction
Tumor Cell-ECM Adhesion
ECM
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Blood Cells
Capillary
GFs
Activated OC
Pln
sRANKL
Embolus
Intravasation
Vein
Pre OC
OPG
RANK
RANKL
ROS
OB
Prostate Tumor in the Skeleton
Tumor Cell-Endothelial Cell Adhesion
Endothelial Cells